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OF

LOGICAL CHEMISTRY

EDITED BY

J. J. ABEL and C. A. HERTER
Baltimore New York

VOLUME III.
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Osborne and Harris[1] obtained similar results with the nucleic acid of the wheat embryo. Although they had at their disposal an almost unlimited amount of material and employed for their experiments specimens of nucleic acid prepared by various methods, they were unable to find either xanthin or hypoxanthin among the hydrolytic products. Moreover, the quantities of guanin and adenin which they did obtain correspond to one equivalent of the former to one of the latter: from this it would naturally be concluded that the two bases are produced from one nucleic acid.

Again, Jones and Whipple[2] found that the nucleic acids of the suprarenal gland and the pancreas yield both guanin and adenin but neither xanthin nor hypoxanthin. Here also it was observed that the ratio of the quantities of guanin and adenin obtained was a simple multiple of the ratio of their molecular weights. Finally, Levene[3] has recently introduced new methods for the preparation of nucleic acids and has been able to make a very accurate examination of the decomposition products of a large number of substances of this class. His results are uniform: guanin and adenin were always present but neither xanthin nor hypoxanthin were ever found even in traces, although in several instances special care was employed in the analysis to find these two bases.

From the results stated we would be justified in concluding that nucleic acids do not contain groups which give rise directly either to xanthin or hypoxanthin and that the occurrence of these two bases is referable to guanin and adenin groups in nucleic acids. The presence of xanthin and hypoxanthin in glands is in all probability due to the action of two ferments in whose presence the two bases are formed by the removal of amido groups from guanin and adenin, respectively. If xanthin and hypoxanthin were once formed in a gland in this way, they would probably contaminate the nucleic acid obtained from the gland, and appear among the hydrolytic products. This seems to be one reason for the finding of the oxypurins by the earlier

[1] *Ibid.*, xxxvi, p. 85.

[2] *Amer. Journ. of Physiol.*, vii, p. 423.

[3] *Zeitschr. f. physiol. Chem.*, xlvii, p. 140; *ibid.*, xlvi, p. 155; *ibid.*, xlv, p. 370.

investigators. A second reason is to be found in the fact that in splitting nucleic acids, hydrolytic agents may have been employed which, while they have little if any power to convert the amido purins into oxypurins, may nevertheless be able to remove amido groups from the nucleic acid or form some intermediate hydrolytic product, thus changing a guanin group into a xanthin group so that xanthin will be found among the final products. However this may be, the fact remains that nucleic acid prepared free from impurities and submitted to hydrolysis under proper conditions, will yield both guanin and adenin but neither xanthin nor hypoxanthin. The matter has become of considerable interest to us since we have found that as a general proposition, both xanthin and hypoxanthin are found among the products of the self digestion of glands while guanin and adenin are absent. This apparent anomaly was explained by the discovery in the tissues of two ferments: one of which (guanase) causes the conversion of guanin into xanthin but is without action on adenin; the other (adenase) causes the corresponding transformation of adenin to hypoxanthin; but is without action on guanin.[1] Our first reported experiments on this subject were made with the thymus gland[2] which contains both ferments and which therefore on self-digestion gives rise to xanthin and hypoxanthin. After the lapse of several years since the publication of this work, when the existence of the two ferments has many times been proven, a contribution by Steudel would make it appear that thymus nucleic acid yields all four of the nuclein bases. While we do not consider that Steudel's work has any bearing on the conclusions which we formerly drew concerning the ferments of the thymus, it is a matter of considerable interest to know whether this nucleic acid which certainly differs from other nucleic acids in its conduct toward hydrolytic agents also differs from them in reference to its purin groups; that is, whether the xanthin found among the decomposition products is really due to the presence of a xanthin group in the nucleic acid or merely to the action of reagents on a guanin group.

[1] Jones and Partridge: *Zeitschr. f. physiol. Chem.*, xlvii, p. 343; Jones and Winternitz: *ibid.*, xliv, p. 1.

[2] Jones: *ibid.*, xli, p. 102.

The Method of Hydrolysis

As already stated, Kossel obtained only adenin by hydrolysis of thymus nucleic acid but afterward Kossel and Neumann obtained also a small amount of guanin. In the more recent work of Steudel,[1] three methods of hydrolysis were employed:

I. With iodide of phosphorus.
II. With hydrochloric acid and stannous chloride.
III. With dilute sulphuric acid.

The results of this work are given in the following table. The numbers express amounts of nitrogen in percentages of the entire nitrogen of the nucleic acid employed.

	I.	II.	III.
Guanin	3.61	3.15	10.07
Xanthin	6.74	?	?
Adenin	13.45	4.76	16.39
Hypoxanthin	5.20	?	?

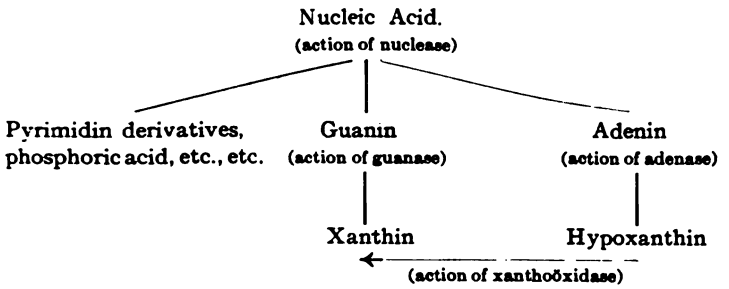
In describing Experiments II and III, Steudel makes no mention of xanthin and hypoxanthin but by comparison of the figures in the three columns it is clear that the bases must have been found. Moreover, we satisfied ourselves that both xanthin and hypoxanthin are formed in the hydrolysis of thymus nucleic acid by Steudel's third method. It is, therefore, apparent that in the case of this nucleic acid the products depend quantitatively within wide limits upon the hydrolytic agent employed and it therefore becomes necessary to study the products obtained under conditions which admit of a transformation neither of products formed nor of groups in the nucleic acid. Fortunately we have of late come into possession of a method which meets these requirements and which may be briefly described as follows:

There is almost universally distributed in animal organs a ferment (nuclease) which in faintly acid media effects the hydrolysis of nucleic acids with the formation of the amido purins.[2] Under the influence of two other ferments usually present the

[1] *Zeitschr. f. physiol. Chem.* xlii, p. 165; *ibid.*, xliii, p. 402; *ibid.*, xlvi, p. 331.

[2] Iwanoff: *Zeitschr. f. physiol. Chem.*, xxxix, p. 31.

amido purins are converted into the corresponding oxypurins.[1] Of rarer occurrence is a ferment (xanthoöxidase) which in the presence of oxygen brings about an oxidation of hypoxanthin to xanthin and this in turn to uric acid.[2] It is, therefore, clear that in the action of an aqueous extract of a gland on a nucleic acid xanthin may be formed directly from guanin or indirectly from adenin.



In this connection the pig's spleen is exceptional.[3] It contains nuclease and adenase, but neither guanase nor xanthoöxidase. Thus the ferments of this gland, while capable of decomposing nucleic acid, cannot cause a formation of xanthin either from guanin or from adenin. Hence we have in an aqueous extract of this gland a means of hydrolyzing a nucleic acid at the body temperature in a fluid that is practically neutral and at the same time free from conditions which could produce xanthin except in so far as xanthin groups are present in the nucleic acid molecule.

The Action of an Aqueous Extract of Pig's Spleen on Thymus Nucleic Acid.

Thymus nucleic acid was used in the form of its sodium salt which was prepared by the method of Neumann[4] and consisted of a mixture of the gelatinous and the non-gelatinous compounds.

[1] Jones and Austrian: *ibid.*, xlviii, p. 111.

[2] Spitzer: *Arch. f. Physiol.*, lxxvi, p. 192; Wiener: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 373.

[3] Jones: *Zeitschr. f. physiol. Chem.*, xlv, p. 84. See also Schittenhelm: *ibid.*, xlvi, p. 354; Jones and Austrian: *loc. cit.*

[4] *Arch. f. Physiol.*, 1899, suppl., p. 552.

The product was free from proteid and contained 14.18 per cent of nitrogen. The solution of ferments was prepared as follows: Pig's spleen which had been carefully freed from external membrane, was ground in a machine to a smooth paste, treated with five times its weight of water and allowed to stand for 36 hours at the room temperature in a well closed vessel with a sufficient amount of chloroform to prevent putrefaction. The fluid was then strained through linen and placed with additional chloroform in one large, well closed vessel from which portions were removed as required. In order to determine the quantities of purin bases produced by the self-digestion of this ferment solution, three portions of 1650 cc. each were treated with 3 cc. of 20 per cent acetic acid and kept at the body temperature for seven days. The products of the digestion were then heated to boiling and the filtered fluid examined for purin bases by the method described below in connection with thymus nucleic acid. As in our former experiments no trace of either xanthin or adenin could be found but guanin and hypoxanthin were both obtained. The former was converted into its characteristic crystalline hydrochlorate; the latter into its nitrate which appeared under the miscroscope as a uniform mass of whetstone crystals, and failed to respond to the color test for xanthin with nitric acid and caustic soda. The quantities of the two salts obtained are given in the following table:

	I.	II.	III.	Mean.
Guanin hydrochlorate	0.188	0.176	0.191	0.185
Hypoxanthin nitrate	0.260	0.269	0.273	0.274

The guanin hydrochlorate was analyzed with the following results:

0.1761 gram lost 0.0285 gram at 105°.
0.1906 gram lost 0.0308 gram at 105°.
0.1587 gram of dehydrated salt required 7.7 cc. sulphuric acid. (1 cc. = .0077 gram N).
0.1472 gram of dehydrated salt required 7.1 cc. of sulphuric acid.

	Theoretical for $C_5H_5N_5O.HCl.2H_2O$.	Found. I.	II.	III.	IV.
$2H_2O$.....	16.11	16.18	16.16	—	—
N.....	37.33	—	—	37.36	37.14

This salt is a most convenient form of guanin both for purification and analysis. It crystallizes from very dilute solutions in 5 per cent hydrochloric acid in clusters of long silky needles: from more concentrated solutions it is deposited in prismatic needles. It it easily soluble in ten parts of hot 5 per cent hydrochloric acid but requires 5000 parts of the acid to effect its solution in the cold and is not removed from its acid solution by animal charcoal: so that it can be decolorized and recrystallized with very small loss of material. The salt has little tendency to lose its water of crystallization on exposure to the air, but suffers loss when kept over sulphuric acid. Several specimens of the substance after exposure to the air during three months were found to contain the required amount of water which was given up sharply at 80°, no further loss occurring at 120°.

The hypoxanthin nitrate was converted into the free base and analyzed.

0.2010 gram required 10.7 cc. of sulphuric acid. (1 cc. = 0.0077 gm. N.)
0.1802 gram required 9.6 cc. of sulphuric acid.

	Theoretical for $C_5H_4N_4O$.	Found. I.	Found. II.
N	41.18	40.99	41.02

Parallel with the three digestions described, three others were carried on with the sole difference that to each of the latter was added before the digestion an aqueous solution of 14 grams of the sodium salt of thymus nucleic acid. When the digestion had continued for seven days, the product was heated to boiling, and the filtered fluid after addition of 10 cc. of 25 per cent sulphuric acid, was evaporated on the water bath to about 250 cc. The acid solution was boiled for ten minutes, diluted with water, and made strongly alkaline with ammonia. The alkaline fluid was treated with an excess of a solution of silver nitrate in ammonia and the precipitated silver compounds of the purin bases were decomposed with hydrochloric acid. The acid solution of the bases was filtered from silver chlorid and evaporated to a syrup for the expulsion of most of the free acid. The residue was treated with hot water and without filtration. The fluid was first neutralized with ammonia and then treated with such an excess

that the fluid contained 3 per cent of the reagent. The material was digested for several hours at 50° and after standing over night at the temperature of the room was filtered. In the filtrate we should be prepared to find xanthin, hypoxanthin and adenin; in the residue, magnesium ammonium phosphate and guanin but no other purin base except in so far as it is included in the precipitate. The residue was treated with 2 per cent ammonia and after digestion at 50° and standing over night the ammoniacal fluid was filtered off and united with the first ammoniacal filtrate.

The mixture of guanin and phosphate was dissolved in hot 1 per cent caustic soda and the guanin precipitated with acetic acid. The base was crystallized from hot 5 per cent hydrochloric acid. On cooling, the solution deposited guanin hydrochlorate in the usual aggregates of feathery needles. Two of the specimens were analyzed.

0.1262 gram lost 0.0203 gram at 105°.
0.1124 gram lost 0.0181 gram at 105°.
0.1086 gram required 4.4 cc. of sulphuric acid. (1 cc. = 0.0077 gm. N.)
0.1435 gram required 5.8 cc. of sulphuric acid.

	Theoretical for $C_5H_5N_5O.HCl.2H_2O$.	Found. I.	II.	III.	IV.
$2H_2O$	16.11	16.09	16.10	—	—
N	31.33	—	—	31.19	31.12

The ammoniacal filtrate from guanin was treated with silver nitrate in ammonia and the silver compounds of the purin bases decomposed with hydrochloric acid. The acid filtrate from silver chlorid was evaporated to dryness, and the addition of water with subsequent evaporation repeated until the free acid had been expelled. The crystalline residue dissolved easily in water at 40°, leaving only a trace of brown flocculent material, so small that it could not be collected for a color test. *Xanthin was therefore not present even in traces.*[1] A small portion of the fluid was tested for adenin with picric acid. As this test proved negative the hypoxanthin was isolated in the usual way and finally converted into the nitrate. This appeared as a perfectly uniform mass of whetstone crystals which were free from xanthin nitrate

[1] Krüger and Solomon: *Zeitschr. f. physiol. Chem.*, xxvi, p. 350.

as shown by their failure to respond to the color test with nitric acid and caustic soda. Two of the specimens were analyzed.

0.2131 gram required 11.3 cc. of sulphuric acid. (1 cc. = 0.0077 gm. N.)
0.1808 gram required 9.6 cc. of sulphuric acid.

	Theoretical for $C_5H_4N_4O$.	Found. I.	Found. II.
N	41.18	40.83	40.88

The quantities of guanin hydrochlorate and hypoxanthin nitrate obtained in the three experiments are given in the table below. If we deduct the amount of these constituents obtained from the self-digestion of the ferment solution the difference will represent the bases produced from the added nucleic acid.

	Guanin Hydrochlorate.				Hypoxanthin Nitrate.			
	I.	II.	III.	Mean.	I.	II.	III.	Mean.
From 1650 cc. of ferment solution + 14 gm. nucleic acid	1.258	1.286	1.278	1.274	1.601	1.587	1.694	1.627
From 1650 cc. of ferment solution	0.188	0.176	0.191	0.185	0.260	0.269	0.293	0.274
From 14 gm. nucleic acid	—	—	—	1.089	—	—	—	1.353

By an obvious calculation from these data it will be found that 18½ per cent of the nitrogen of thymus nucleic acid formed hypoxanthin, 18 per cent formed guanin. By comparing these figures with those obtained by Steudel the following points appear:

	By Action of Iodide of Phosphorus.	By Action of $HCl + SnCl_2$	By Action of Boiling H_2SO_4	By Action of Ferments.
Guanin	3.61	3.15	10.07	18.0
Xanthin	6.74	?	?	none
Adenin	13.45	4.76	16.39	none
Hypoxanthin	5.20	?	?	18.5

1. By the action of nuclease, thymus nucleic acid produces no xanthin. The xanthin formed by more violent methods of hydrolysis must therefore originate in guanin groups or in guanin itself.

2. Hydrolysis at high temperatures not only gives results that are misleading but actually destroys a large amount of purin products. This is especially true of guanin. The amount of this base formed by ferment action is nearly twice as great as the sum of guanin and xanthin obtained by hydrolysis with chemical reagents.

3. The quantities of guanin and hypoxanthin (equivalent to adenin) formed by the action of the ferments are as nearly proportional to the molecular weights of the two bases as could reasonably be expected with the use of the methods at our disposal. This constitutes strong evidence that the two bases result from the same nucleic acid.

THE EXCRETION OF BORIC ACID FROM THE HUMAN BODY.

By HARVEY W. WILEY, M.D.

(*From the Bureau of Chemistry, Washington, D. C.*)

(Received for publication, December 15, 1906.)

In the studies which I have inaugurated for the determination of the effect of various substances added to foods on health and digestion, the first substances investigated were boric acid and borax. The object of this investigation was to administer these bodies in small quantities to young men over a long period of time and determine the effect upon health, digestion and the various metabolic processes. Incidental to this investigation, the fate of the boric acid in the human body was studied. It was found that, as had been pointed out by other investigators, by far the larger quantity of the boric acid administered was excreted through the kidneys. There was a marked difference in the action upon the urine between the borax and boric acid. When boric acid was administered the acidity of the urine was considerably increased. When borax was administered, the acidity was diminished, the reaction sometimes becoming amphoteric and even alkaline.

The first experimental work was divided into five series, twelve young men being observed during each of the series. The time of the series varied somewhat in length. The first series began on the sixteenth of December, 1902, and ended on the thirteenth of January, 1903. The second series began on the nineteenth of January, 1903, and ended on the twenty-first of February, 1903. The third series began on the nineteenth of February, 1903, and ended on the nineteenth of March, 1903. The fourth series began on the twentieth of March, 1903, and ended on the twenty-second of April, 1903. The fifth series began on the twenty-fourth of April, 1903, and ended on the twenty-ninth of June, 1903.

In the first series there were administered altogether 150 grams of boric acid, half of it as borax. There were recovered in the urine 124.58 grams, or an average percentage of recovery of 83.05. In the second series the average percentage of recovery was 82.85, the total amount administered being 98 grams and the amount recovered 81.19 grams. In the third series there were administered 132.90 grams and there were recovered 84.90 grams, an average of 63.88 per cent. In the fourth series there were administered 99.50 grams and recovered 82.55 grams, equivalent to 82.96 per cent. In the fifth series there were exhibited 127 grams and recovered in the urine 95.47 grams, an average of 75.17 per cent.

The total quantity of boric acid (and borax as boric acid) administered during the investigation was 607.40 grams, of which there were recovered in the urine 468.69 grams, the average percentage recovered being 77.16.

It having been stated by some investigators subsequent to the publication of these results that the amount of boric acid excreted in the urine as given was too small, a supplementary experimental determination was made with six young men over a period extending from the twenty-first of January, 1905, to the fourteenth of February, 1905. In each case after the cessation of the administration of the substance the tests of the urine were continued, until for several days, by the method employed, no determinable quantity of boric acid was noticed. The percentages of boric acid eliminated in the six cases were as follows:

In No. 1, 83.51; in No. 2, 77.84; in No. 3, 91.55; in No. 4, 87.75; in No. 5, 84.26; in No. 6, 77.38.

The Thompson method[1] employed here was the same as that employed in our previous work.[2] Calcium hydroxid was used throughout and the only departure from the regularly outlined method was in redissolving and precipitating more times in order to free the occluded boric acid from the precipitate. The volume of solution which was obtained was kept constant throughout.

The second method with a few modifications is that of Fendler[3]

[1] Sutton: *Volumetric Analysis*, 8th ed., p. 98.

[2] Bureau of Chemistry, Bulletin 84, pt. 1, "Boric Acid and Borax."

[3] *Zeitschr. f. Untersuch. d. Nahrungs u. Genussmittel*, xi, p. 137, 1906.

and consists essentially of concentrating a portion of the sample in the usual manner, acidifying and immersing the turmeric paper strips in it. After sufficient time has elapsed to allow the liquid to travel up the paper it is compared with strips which have been immersed in a solution whose exact content of boric acid is known.

It is seen that in three subjects of the series there was more than 80 per cent recovered, while in three others of the series there was less than 80 per cent recovered. During this investigation experiments were also made to determine whether any of the boric acid administered assumed a volatile state and escaped through the expired air, or if any of it was found in the respiration. The well known tendency of boric acid to assume the volatile condition, especially when heated with methyl alcohol and some other bodies, led to the supposition that it might be reduced in the system to the form in which it would be volatilized in the expired air. To determine this point the expired air was forced continuously through a solution of lime water for three hours. The lime water was afterward tested for boric acid with a negative result. It appears, therefore, that no appreciable quantity of boric acid escapes through the lungs. On the other hand it was found that very considerable portions of the boric acid are excreted through the perspiration.

From the above data it is seen that the average percentage of administered boric acid excreted in the urine in this series is 83.71, which is considerably greater than that given for the first series of determinations. When the data are looked over, however, it is seen that this discrepancy is easily accounted for. In the first set of investigations there was one series where the proportion of boric acid recovered in the urine was only 63.88 per cent, while in the present series there was one instance where the proportion excreted was 91.55 per cent. If these abnormal results be omitted from each of the investigations, it is seen that they are practically the same. Thus in the first series of observations, made in 1903, the average percentage of borax excreted in the urine, excluding the one phenomenally low case, is 82.15, while in the observations made in 1905, excluding the one phenomenally high case, the average percentage excreted is 81.01.

These data show that the relative quantity of boric acid excreted varies with the individual case and may extend from 63

to 91 per cent of the whole amount ingested. These data seem to establish very conclusively that the average percentage of administered boric acid which is excreted by the kidneys is a little more than 80 per cent.

In the spring of 1906 another series of determinations was made, extending from the fourth to the twenty-second of April. The object of this investigation was to determine the quantity of boric acid secreted in the perspiration, and the examinations of the urine after the exhibition of a small quantity of borax were continued until no further test for boric acid could be distinguished. On the first day of the observation the urine was examined carefully for boric acid. None was found by the usual method of determination and a trace only by Fendler's method. On the fifth of April one gram of boric acid was given and on the sixth of April, two grams.

The examinations by the usual method showed that after the eleventh of April, five days after the administration of the last borax capsule, there was no longer a sufficient quantity of boric acid in the urine to be determined. On the contrary, by Fendler's method there was still estimated to be on the twenty-second of April 0.002 per cent. The total percentage of the 3 grams administered which was excreted in the urine in this case was 65.9. This indicates that there was a retention of a very large proportion of the boric acid administered in the system, and that this retention was of somewhat indefinite duration, small quantities of boric acid being given off in the urine 16 days after its administration. A second subject, treated exactly in the same way, showed a percentage excretion of boric acid in the urine, by the usual method, of 71.3. There was still an appreciable quantity of boric acid in the urine by the Fendler method on the sixteenth day after the administration of the capsules. A third subject, treated in exactly the same way, showed an excretion of 82.96 per cent of boric acid in the urine and an estimated amount of 0.003 per cent in the urine on the sixteenth day after administration, by the Fendler method.

The average quantity of boric acid excreted in the urine in these three instances is 73.3, showing that when only a small quantity of boric acid is administered a very much smaller percentage of it is recovered in the urine than when the administra-

tion is continued over a long period, thus indicating very plainly the tendency to accumulate the boric acid in the system. This fact is accentuated by the further observation that even after sixteen days an appreciable quantity of boric acid was still excreted in the urine.

Excretion of Boric Acid in the Feces.

Considerable quantities of boric acid when administered as described in the preceding pages, are excreted in the feces, but the total quantity is so small, compared with that eliminated in the urine, as to be of little importance from a merely chemical point of view.

In the case of Subject No. 1 in the series of April, 1906, 1.12 per cent of the administered borax was recovered in the feces. In the case of No. 2, 0.78 per cent, and in the case of No. 3, 0.68 per cent. The average shows 0.86 per cent of the borax administered excreted in the feces. This number must be regarded as a minimum percentage of excretion, since only 3 grams of borax were administered in these cases, altogether, and the observations continued until no appreciable quantities of boric acid were found in the feces. Traces, however, still remained after the above observations were concluded. It is evident, therefore, that a weighable portion of borax, administered as above described, is excreted in the feces, probably only about 1 per cent of the total quantity, where only a small quantity is exhibited for a short time.

Excretion of Boric Acid in the Perspiration.

It is evident from a general idea of the method of excreting boric acid that weighable portions of it should be found in the perspiration. This theoretical consideration was verified on several occasions during the progress of the work in the earlier periods.

In the spring of 1906 it was decided to determine, if possible, quantitatively the amount of boric acid excreted in the perspiration. This was the principal object of the series of the spring of 1906, with the three men above mentioned. The method of determination, while perhaps not capable of absolute exactness, is one

which at least determines approximately the proportional amount of boric acid recovered. The three men above mentioned, after having taken 1 gram of borax on the fifth of April, and 2 grams on the sixth, one in the morning and one at noon, were conducted to the hot room of a Turkish bath in the afternoon on the sixth of April. Before entering, they were carefully washed in distilled water and thoroughly dried with an extracted towel. They were then placed in a suit of thick woolen under-clothing which had been previously washed, steeped in distilled water and extracted in this way until no further extract was removed, and then dried. They remained in the Turkish bath at a temperature of from 130° to 135° F. for an hour and thirty minutes. The suit of under-clothing was then removed and the men, standing in a basin, were thoroughly sponged with distilled water, the washings saved and evaporated, and the residue added to the extract from the woolen suit. After concentration, the quantity of boric acid recovered was determined. The quantity of boric acid recovered in the perspiration in the case of No. 1 was 0.0299 gram, equivalent to 1 per cent of the whole amount exhibited. The quantity recovered in the case of No. 2 was 0.0311 gram, equivalent to 1.03 per cent of the amount exhibited. The quantity recovered in the case of No. 3 was 0.0777 gram, equivalent to 2.59 per cent of the quantity exhibited. In the case of No. 3 it should be observed that the washings in distilled water were accidentally lost, so that the amount represented as recovered was simply that derived from the woolen suit.

The average percentage of boric acid recovered, based upon the amount exhibited, is 1.54. It is fair to assume that the amount of perspiration during the hour and a half spent in the hot room of the Turkish bath is practically equivalent to that of 24 hours of ordinary temperature, so that roughly we may assume that the average percentage of the exhibited boric acid excreted in the perspiration of 24 hours would be 1.54.

No determination was made of the quantity of boric acid which could be secured on subsequent days, although it is evident that as long as it remains in the system and is excreted in traces in the feces and in appreciable quantities in the urine, traces would also be found in the perspiration.

The general conclusion derived from these experimental data is

that the total quantity of boric acid excreted in the feces and perspiration is not much if any over 3 per cent of that administered during the ordinary period of observation. It is evident, therefore, that even including these quantities with those which are excreted in the urine, not over 85 per cent of the total amount of boric acid exhibited in these experiments is in the three excretions mentioned.

Excretion of Boric Acid in Milk.

From theoretical considerations it is evident that a substance of the character of boric acid would be found also in the milk as well as in the other excretions of the body. Accordingly arrangements were made with a hospital to examine the milk of young mothers shortly after childbirth. The milk was secured by the attendants of the hospital in the usual way. Preliminary examinations were made in all cases to establish the presence of boric acid in the milk. In only one case was it found in the preliminary examination and this was evidently due to the exhibition thereof at some time prior to the entry of the patient into the hospital.

The samples were obtained by L. F. Kebler and J. K. Haywood, of the Bureau of Chemistry, and the determinations were made by Rudolph Hirsch and W. B. Smith.

In the case of the first subject the lacteal secretion was so diminished before the time of the preliminary exhibition of the boric acid as to render the experiment of little value. After five days of preliminary examination, begun on the twenty-sixth of June, 1906, 1 gram of boric acid was administered on the first of July. The milk which up to this time had given no trace of boric acid, on the first examination thereafter showed a decided trace which, estimated colorimetrically, was equivalent to about one part in 100,000 parts of the milk. After the second of July the flow of milk became so diminished in quantity that the experiment was discontinued.

In the case of the second subject the preliminary examination of the milk was finished on the twenty-seventh of May, 1906. On the twenty-eighth of May 1 gram of boric acid was administered, the same amount on the twenty-ninth, 2 grams on the thirtieth, and 3 grams on the thirty-first, the first of June, and second

of June. In this case no trace of boric acid was found in the milk until the first of June, when a mere trace was discovered. On the second of June, the last day of the administration of the preservative, the quantity excreted in the milk was estimated at one part in 165,000. On the following day, being the first succeeding the cessation of the administration of the boric acid, the amount in the milk had fallen to a mere trace. On the fourth, fifth and sixth of June there was not even a trace. Unexpectedly on the seventh of June, five days after the cessation of the administration of the boric acid a very large quantity was found in the milk, namely, one part in 25,000. On the eighth of June there was one part in 60,000; on the ninth and tenth of June, one part in 90,000, respectively. In this instance it is seen that with the exception of the first day it was not until long after the administration of the boric acid that it appeared in the milk. Immediately after ceasing the administration of the boric acid it disappeared from the milk and did not reappear until the fifth day, when it was present in its maximum quantity, diminishing on successive days until the end of the observation, at which time the amount present in the milk was one part in 90,000.

In the third subject the preliminary examination, which ended on the twenty-seventh of June, having extended over four days, exhibited a somewhat remarkable phenomenon, namely, that on the first day of the preliminary examination, the largest quantity of boric acid which was found at any time in the milk was indicated, namely, one part to 3500. On the two succeeding days the amount dropped to a trace while on the twenty-seventh of June, the last day of the preliminary examination, not even a trace was found. One gram of boric acid was exhibited on the twenty-eighth and twenty-ninth of June, 1½ gram on the thirtieth of June, and 2 grams on July 1 and July 2. On the twenty-ninth of June the amount of boric acid in the milk was one part in 100,000. On the thirtieth of June and first of July there was only a trace of boric acid in the milk. On the second of July, which was the last day of the administration of the boric acid, there was one part in 100,000. On the third of July, which was the last day of the observation, there was one part in 25,000.

These data indicate that appreciable quantities of boric acid administered to the mother are found in the lacteal secretion.

The quantity is quite variable and increases or decreases without much relation to the exact date of administration of the preservative. It is evident that the residue of boric acid which is stored in the body may at any time be expected to appear in the milk. Properly this investigation should have been completed by a study of the animal body itself after the administration of boric acid for a certain period to determine in what organs the part which escapes excretion is principally stored. Theoretically, from the results of the metabolic experiments, a large part of it would be found in the bones, or other phosphatic tissues, since it was seen that the administration of the boric acid largely increased the excretion of phosphorus. It is our intention in the near future to complete the experiment by feeding animals borax or boric acid for a period of time and then examining their bodies to determine the quantity of borax stored and its distribution.

CREATIN AS A BRAIN STIMULANT.

By S. S. MAXWELL.

(*From the Rudolph Spreckels Physiological Laboratory of the University of California.*)

(Received for publication, January 1, 1907.)

In a former paper[1] I believe that I have proved that the substances which we may consider specific nerve stimulants, namely, those salts whose anions tend to precipitate calcium, produce very prompt, in fact almost instantaneous, effects when applied directly to the white matter of the corona radiata of the motor areas; and, further, that no such effects are produced when these salts are applied to the gray matter.

A number of years ago Landois[2] had shown that muscular tremblings and cramps are produced by the application of creatin to the motor areas of the cortex. In his experiments the cramps occurred only after a relatively long period, seven or eight minutes to three-fourths of an hour, and when once established they often lasted for many hours.

The questions now arose, first, whether the action is on the white or on the gray matter, and, second, if on the latter, why the latent period is so long?

When *powdered* creatin was applied to the cortex of the motor areas I obtained results agreeing in every essential detail with the description given by Landois. When, however, a saturated *solution* of creatin was applied to the brain surface the latent period was in every instance much longer and the stimulating effects were much less marked. The following is the record of a typical experiment:

Rabbit: right motor area exposed and determined by electrical stimulation. Surface kept continuously moistened with saturated solution of creatin warmed to body temperature.

10:40 Began applying the solution.
10:50 Fright and slight hæmorrhage.

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[1] This *Journal*, ii, p. 183, 1906.

[2] *Deutsch. med. Wochenschr.*, xiii, p. 685.

11:00 Forepart of body inclines slightly to left. A few chewing movements.
11:07 Slight fits of trembling in muscles of neck and of hind limbs. Soon discontinued.
11:15 Slight chewing movements, with momentary tremblings of muscles of neck and left foreleg.
11:27 A few chewing movements. Slight fits of quivering, mainly of muscles of left side, have occurred at intervals for the past ten minutes.
11:28 Discontinued the solution and applied *powdered* creatin.
11:36 Severe tremblings.
11:40 Epileptiform twitchings of left foreleg.
11:43 Left hind leg also twitching.
11:45 Paroxysm has become general.

In the above experiment the solution had been applied for twenty minutes before any effect could be perceived and the effect of forty-eight minutes' continuous application was comparatively slight, and did not apparently intensify the results of the subsequent use of the powdered substance.

In my previous experiments[1] I had found that when the calcium precipitants were injected to the level of the white matter stimulation usually occurred very promptly. Saturated solutions of creatin injected in the same way gave no indication of stimulation of the white matter. Occasionally in my notes occur such expressions as "twitching of nose," "chewing movements a few seconds after," but not oftener than the accidental coincidence of these movements could be expected. Ordinarily the record is "no effect observable." No limb movements were seen at any time. It must be remembered, however, that it is not possible to introduce more than a few drops of solution in this way without doing so much mechanical injury as to render the results untrustworthy. Moreover, the experiments described above on application of creatin to the brain surface show that the degree of concentration has a very great effect upon the results and point to a mass action. One could hardly expect, then, that the injection of a few drops of solution, which would necessarily be greatly diluted by the fluids contained in the tissues, could give any definite results, and it would not be safe to conclude from evidence obtained in this way that creatin can not stimulate the white matter directly.

[1] *Loc. cit.*

This question can, however, be approached in another way. The salts which I have found to stimulate the white matter of the corona radiata, are those which were already known through the work of Loeb to be powerful excitants of the peripheral nerves. For this reason I investigated in the following way the effect of creatin solution on the sciatic nerve of the frog:

Nerve-muscle preparations were suspended in a moist chamber in such manner that any contractions which might occur would be recorded on a slowly moving drum. The creatin solution was held in a bent glass tube so placed that the entire nerve could be immersed in it, especial care being taken that the part of the nerve nearest the muscle should have no chance to suffer from drying. In every instance a control experiment was made with the nerve of the companion nerve-muscle preparation immersed in $\frac{M}{8}$ sodium chlorid, it having been shown by Loeb[1] that this solution is without stimulating effect on nerve.[2] In part of these experiments I used a saturated watery solution of creatin; in others I added to the saturated creatin solution sodium chlorid sufficient to secure an osmotic pressure practically equal to that of the body fluids. The results of these experiments were absolutely uniform. Neither contractions nor changes of tonus of the muscle were produced by the creatin solution nor by the mixture of the creatin and sodium chlorid solution. Powdered creatin strewed along the nerve and upon its cut end was equally without effect.

It follows from these experiments that creatin does not excite

[1] Loeb: *Festschrift für Fick*, Braunschweig, 1899, p. 118.

[2] Mathews reports that after a latent period of one hundred and sixty to two hundred and forty minutes rhythmical contractions appear in a muscle whose nerve is immersed in an $\frac{M}{8}$ NaCl solution (*Amer. Journ. of Physiol.*, ii, p. 455). In the course of a large number of these experiments I saw contractions only in two or three exceptional cases. On the other hand I have frequently, after a nerve had lain for from four to six hours in a pure $\frac{M}{8}$ NaCl solution without causing a single twitch, transferred it to an $\frac{M}{8}$ citrate or oxalate solution and have then obtained a perfectly characteristic series of contractions. It seems probable that the exceptional twitches which may occur with the nerve in $\frac{M}{8}$ NaCl are due either to erroneous methods of experimentation or to secondary changes of some kind within the nerve, and not directly to chemical stimulation by sodium chlorid.

the peripheral motor nerves and the probabilities are that it does not stimulate the white matter of the brain.

If the action of the creatin is upon the gray matter, as apparently it is, we may now ask why its effects appear so tardily as compared with the effect of applying the nerve-stimulating salts to the white matter?

Since powdered creatin is so much more effective than the solution and since, as I have found,[1] epileptiform contractions may be produced by applying crystals of cane sugar to the brain surface, one might suppose that creatin acts by the extraction of water from the tissues. That this is not so is indicated by the following facts: Creatin is not markedly hygroscopic. The quantity of sugar necessary to produce cramps is enormously larger than that of creatin. A saturated solution of the creatin employed in these experiments, could not according to freezing point determinations, give rise to an osmotic pressure greater than that of an $\frac{M}{13}$ sodium chloride solution. And finally powdered creatin applied directly to the motor nerve does not stimulate.

That the effects of the creatin appear so slowly and that they depend upon concentration to so marked a degree indicates that they are the result of chemical changes brought about in the gray matter.

There are apparently two classes of substances which act as brain stimulants. The first class includes such substances as sodium citrate, oxalate, etc. These excite the white matter only, but they do this promptly, almost instantaneously, and their action is probably a direct one. The second class of which creatin is an example, produce effects only after a relatively long latent period. They do this apparently by bringing about chemical changes in the gray matter. Among the by-products of these reactions may be small quantities of the specific nerve stimulants and these when they have accumulated may give rise to the stimulation. In other words substances like creatin probably do not stimulate directly but bring about secondary chemical changes to which the excitation is due.

[1] *Loc. cit.*

THE FORMATION OF GLYCOGEN IN MUSCLE.

Plate I.

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(Received for publication, December 28, 1906.)

It would appear from theoretical considerations that muscle tissue should be incapable of forming glycogen from a disaccharid.

As has been shown by a number of observers,[1] the amount of inverting substance present in muscle or blood is small, or is entirely absent. The injection experiments with saccharose,[2] and perfusion experiments with the liver[3] also tend to show that saccharose is quite incapable of being used by the organism as a direct glycogen former. Moreover, the direct transformation of a disaccharid to a polysaccharid without the preliminary inversion to a simple hexose has not, as far as we are aware, been shown to take place.

Nevertheless E. Külz,[4] in a series of experiments undertaken to decide this question some years ago, obtained results which in effect showed that saccharose was indeed a direct former of glycogen in muscle.

It seems that the work of Külz, has never been repeated, although the anomalous character of the findings as recognized by him, and he asked that his observation be tested by other observers. On his authority the statement that saccharose is a direct former of glycogen has passed into the text-books, and Hammarsten, among others, mentions saccharose as an immediate antecedent in the formation of glycogen. On reference to Külz's original experiments, it will be seen that the results on which he bases his statement are by no means unequivocal. Three experiments

[1] Tebb: *Journ. of Physiol.*, xv, p. 421, 1893.

[2] Bernard: *Leçons sur le diabète.*

[3] Pflüger: *Das Glykogen*, p. 205; see also Jappelli and D'Errico: *Atti Real. Accad. di Med. di Napoli*, 1903, cited by Moscati, *Zeitschr. f. physiol. Chem.*, l, p. 90, 1906. These authors believe that saccharose is transformed into glycogen by injection into the portal vein.

[4] E. Külz: *Zeitschr. f. Biol.*, xxvii, p. 237, 1890.

only are reported which confirm his conclusions, and these are not given with very great confidence. For this reason we have thought it advisable to repeat Külz's experiments with improved technique, and try if possible to obtain his somewhat anomalous results.

Through the exhaustive work of Pflüger[1] we are now in possession of a method which permits the more accurate determination of glycogen than the Brücke-Külz[2] method employed by Külz,[3] and using a method of perfusion which will be presently described, we believe we have been able to keep a muscle in a surviving condition for a period of time sufficient to decide the question. At the same time we have been able to fulfill certain experimental conditions which are necessary to render the results trustworthy.

The methods used by Külz were two. In the first, a limb was perfused with blood to which 0.1 per cent of saccharose had been added, and glycogen estimations were carried out in the limbs before and after perfusion, using the unperfused limb as a control. This method is faulty, in that one is never certain that the glycogen destruction which is continually taking place is going on more rapidly or more slowly than the glycogen formation by the sugar. It is quite possible to have a formation of glycogen which would be completely masked by the simultaneous destruction which is taking place. It will also be seen that the figures as presented by Külz do not differ markedly from the differences which Cramer[4] obtained in his analysis of symmetrical muscles, using the Brücke-Külz method employed in the work under discussion. The objections to this method were realized by Külz and he accordingly changed his experimental conditions to meet the difficulty.

The second method which was used was the perfusion of two symmetrical limbs of the same animal, one with blood containing saccharose, the other with blood containing no foreign sugar. Symmetrical muscles were analyzed. It is the results obtained

[1] Pflüger: *Das Glykogen*, p. 67.

[2] Brücke: *Sitzungsber. d. Akad. d. Wiss. Wien.*, Abth. 2, p. 63, 1871.

[3] Külz: *Zeitschr. f. Biol.*, xxii, p. 191, 1886.

[4] A. Cramer: *Zeitschr. f. Biol.*, xxiv, p. 70, 1888; Aldehoff: *ibid.*, xxv, p. 147, 1889; see also Pflüger: *Das Glykogen*, p. 178.

from this series of experiments on which Külz bases his conclusions.

This method, suitably changed, appeared to fulfill more closely the experimental conditions, and was the one chosen by us.[1]

There are two points which Külz does not appear to have taken into consideration, and which may have some bearing on his results. The perfusion of a limb with blood which has not been properly arterialized does not conform to the state of affairs obtaining under normal conditions. The perfusing fluid was also run in at constant pressure. This while not directly affecting the results has unquestionably an influence on the metabolism of other organs, as Sollman[2] has shown in the case of the kidney. For that reason, we have performed the experiment, using intermittent pressure. In using the lung itself to arterialize the blood instead of the less efficient mechanical oxygenation, one may be guided to some extent by the recent experiments of Riehl,[3] who has shown that lung tissue is incapable of inverting lactose.

The method which Külz used for analyzing the muscle is also very seriously open to question. This is especially the case as in the present instance where the amounts of glycogen are small. In the course of a large number of controls which were undertaken to test the accuracy of the Pflüger method, we convinced ourselves of its entire suitability for the estimation of small quantities of glycogen.

At the outset of this work, it was realized that a perfusion apparatus, fulfilling the conditions of efficient arterialization and certainty of action was absolutely necessary. A number of

[1] One of us, with Dr. B. J. Dryfuss, has attempted the perfusion, by quickly extirpating all the organs of the abdominal cavity. The liver was ligated off piece by piece. Practically no liver tissue was left. There was very little hemorrhage. One iliac artery was then ligated. Artificial respiration was employed, and the animal kept warm on a hot plate, additional heat being supplied by electric lamps. The animal survived over two hours. It was found that the amount of glycogen in the ligated leg was almost unweighable, showing the complete disappearance of the saccharid from ligation. Our principal object in this experiment was to obviate the difficulty of using defibrinated blood. See Barcroft and Brodie, *Journ. of Physiol.*, xxxii, p. 19, 1904; Pfaff and Vejux-Terode: *Arch. f. exp. Path. u. Pharm.*, xlix, p. 324, 1903.

[2] Sollman: *Amer. Journ. of Physiol.*, xiii, p. 253, 1905.

[3] Riehl: *Zeitschr. f. Biol.*, xlviii, p. 309, 1906.

preliminary experiments lead to the adoption of the apparatus described below, which has accomplished admirably the purpose for which it was designed. We also made experiments in mechanically perfusing one limb, using the unperfused limb as a control. It was soon found that the rapid destruction of glycogen in the unperfused limb which sometimes occurred with rigor did not permit of its use.

Kisch[1] has recently examined the destruction of glycogen after death, and has shown that the process takes place with decreasing velocity, owing possibly to a using up of the glycogen-splitting ferment. In the course of this work, we have been able in part to confirm his results. We are also able to add that the destruction of glycogen seems to be dependent on factors of which very little can be conjectured. It was found that in experiments taking place under what appear to be identical conditions, one obtained a complete disappearance of glycogen from the ligated limb, while in the second experiment, the amount of glycogen from symmetrical muscles of perfused and unperfused limbs was identical. That this is not due to disappearance of glycogen in the perfused limb is shown by the high amount of glycogen which is often obtained under these conditions.

In designing this experiment, it was necessary to have an apparatus which possessed two completed systems for perfusing and arterializing each limb. The apparatus is a combination of that used by Embley and Martin[2] with the chamber for containing the limbs and lungs and for keeping the blood and organs at constant temperature as designed by Brodie.[3]

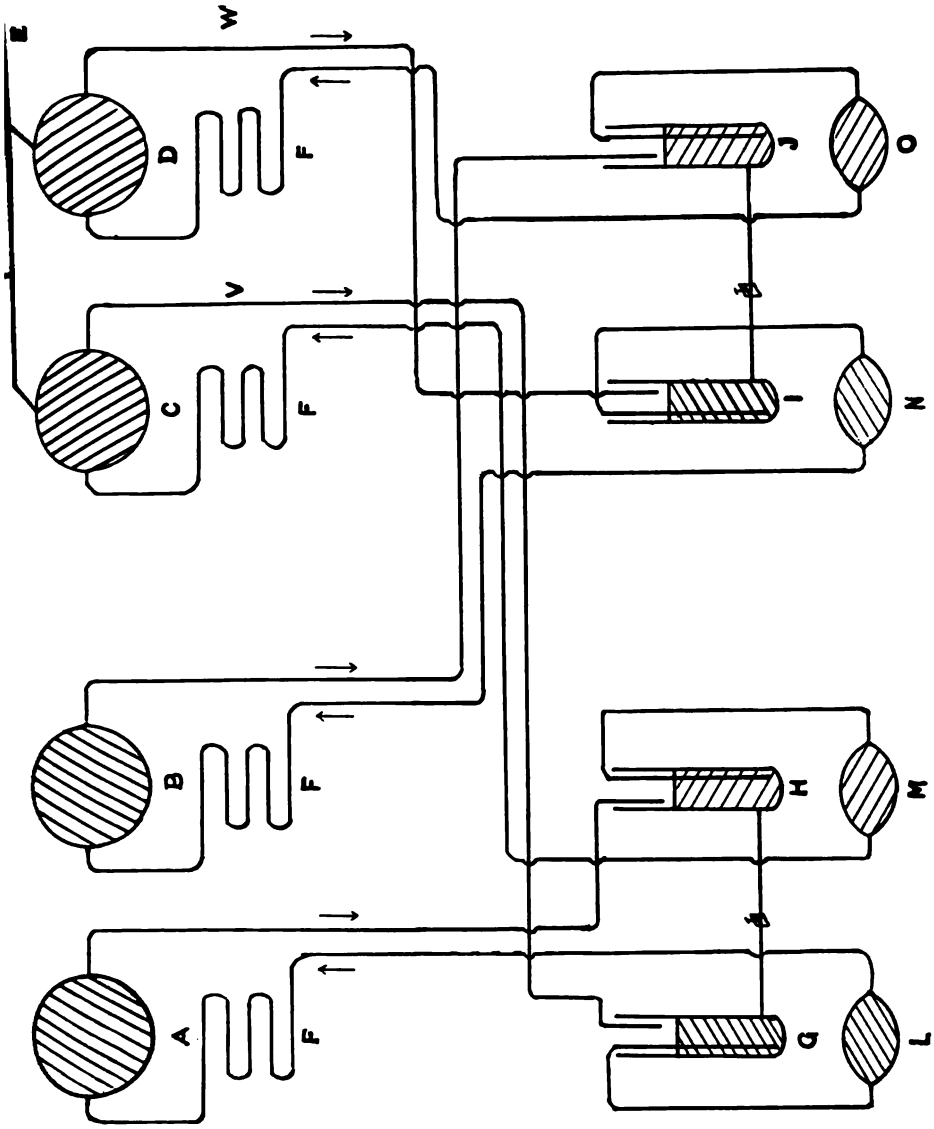
The accompanying plate (I) gives the general arrangement of the apparatus, while the following schematic diagram explains the individual parts of the apparatus.

Description of Diagram. The pumps representing the right and left hearts are formed from ordinary syringe bulbs, *L*, *M*, *N*, *O*, with hard rubber seat valves. *L* and *N* are the right ventricles, pumping blood to the two pairs of lungs, *C* and *D*, which are supplied with air through the tube *E*. After the blood has been

[1] Kisch: *Beitr. z. chem. Physiol. u. Path.*, viii, p. 210, 1906.

[2] Embley and Martin: *Journ. of Physiol.*, xxxii, p. 147, 1904.

[3] We wish to acknowledge the great assistance which one of us received in the technique of perfusion from Prof. T. Gregor Brodie.



PERFUSION APPARATUS.

arterialized, it returns through *V* and *W* to glass receivers *G* and *I*. Thence it is pumped by means of the left hearts *L* and *M* to the respective limbs *A* and *B*. Returning, deprived of oxygen, it reaches the receivers *H* and *J*, and again completes a cycle.

The coils *F* are beneath the organs to be perfused, and are completely submersed in water at body temperature. By means of the connections between the reservoirs *G* and *H*, and *I* and *J*, the levels in the venous and arterial reservoirs are kept equal. At the same time the flow from one reservoir to another is insufficient to mix the two bloods appreciably. Between the heating coils and the organs small air traps are interposed, which are not shown in the figure.

The heating tank is 107 cm. long, 35 cm. wide and 41 cm. deep. The upper part contains two separate trays pierced at the bottom with four holes 2.5 cm. wide, flanged to admit of being closed with two holed rubber stoppers, through which the artery and vein for one organ pass. The water which fills the tank does not enter the trays, but is in contact with the bottom, and supplies the necessary heat to the organs. If the temperature is not sufficiently high in the chambers, additional heat is furnished by electric lamps, which are turned off and on at will.

The system of cams, which is shown in the photograph, and also extremely well in the diagram in Embley and Martin's article, permit of a very accurate adjustment of the flow to any of the organs. It will be observed that instead of the two cams and compressors used by Embley and Martin, we have used four.

The Operation. An animal is killed by medulla puncture, and bled at once from the carotids. The blood is defibrinated, and filtered through glass wool. A second dog is killed in a similar manner, bled and the blood added to that of the first dog. The lungs and hearts are removed from both. The hearts are opened, and very large glass cannulas with heavy shoulders are introduced into the pulmonary arteries and veins through the right ventricles and left auricles. The cannulas are securely tied in, and the excess of heart muscle cut away. Three-way cannulas are placed in the tracheæ. The lungs are then placed in warm saline solution till ready to be used.

The abdomen of the second dog is then opened, and cannulas introduced into the right and left iliac arteries and veins. In

order to prevent collateral circulation as much as possible, ligatures are tied around the profunda arteries and veins just beyond the origin of the iliacs. The limbs are severed from the rest of the body, the bladder tied off and bleeding points stopped with ligatures or with hæmostats. All the cut surface of muscle and skin are cauterized thoroughly, by brushing lightly with a blowpipe flame. The spinal canal is plugged with modeling wax. The amount of capillary oozing which takes place from the limbs is small.

In some few instances we have been able to detect a certain amount of collateral anastomosis, but there were few of these anomalous cases.

After joining up the arterial system with the organ, being careful to exclude air, and removing clotted blood with a small feather, the pumps are separately set in motion by hand, and the blood flowing from the iliac veins and pulmonary arteries collected in basins. About 150 cc. of blood are washed through. This is defibrinated, filtered through glass wool and returned to the proper receiver. In this way the organs are washed out with defibrinated blood, and one has no further trouble from intravascular clotting.

The cams are adjusted, so that each heart makes about 70 beats per minute. The respiration pump inflates the lungs about 25 times per minute. By means of the adjusting screws, the flow to the organ is regulated so that limb and lung receive the same quantity of blood.

The sugar solutions added were isotonic with blood. In every case where sugar solutions were added, an equal amount of isotonic salt solution was added to the control side. In this way we believed we kept the conditions on the two sides more closely alike than if the control side was allowed to receive undiluted blood.

Perfusion was continued for two hours, after which symmetrical muscles were removed from the two limbs as quickly as possible for analysis.[1]

[1] It may be noted that it was found to be extremely difficult to filter the alkaline solution of muscle clear, and it was found preferable to let it stand a day or more for sedimentation to take place: this very greatly facilitates the subsequent portion of the analysis, and no destruction of glycogen occurs even on prolonged standing, of which we satisfied ourselves by experiments.

Before undertaking this work it was necessary to repeat some of the experiments which had been performed to determine the difference which might exist in the glycogen content of symmetrical muscles. This question has been inquired into a number of times since Cramer took the matter up at the suggestion of Külz. The following are some of the result which we obtained.

We give but one of several sets of results which all show approximately the same difference for symmetrical limbs.[1]

TABLE I.

	Glycogen.		
	Fore limb	Hind limb	Pectoral
Right	0.523	0.570	0.533
Left	0.516	0.561	0.526

The difference in the content of glycogen of symmetrical muscles would appear from our results to be much less than that observed by other investigators. Recent observations on the glycogen content of the liver tend to show that the difference in concentration is also less than that heretofore believed to be the case.

The Perfusion of Muscle with Blood Containing Saccharose.

The following table (II) gives the results which we have obtained by the method above described.

As will be seen, the perfusion of a muscle with a solution of saccharose in no case led to an increase in the glycogen content of the side through which the sugar solution was perfused. A number of experiments with a single perfusion, after ligation of one side are not detailed. The loss of glycogen on the ligated side during the two hours of the experiment is sometimes quite large. This is not always the case, however, and we have sometimes observed as great an amount of glycogen in the non-perfused side as in the perfused. The reason for the difference in individual experiments we are at a loss to explain.

[1] These analyses (for which we are indebted to Dr. B. J. Dryfuss) were made by a method similar to that described by Ivar Bang (*Festschrift für Olof Hammarsten*, 1906), in which a centrifuge is used to separate out the glycogen. This method will be found much more convenient than the usual procedure of filtration and washing. Duplicate analyses are very satisfactory.

TABLE II.

Experiment.	Saccharid.	Blood.	Difference.	Difference.	Saccharid.	Blood.	Time.	
				Per cent				
I	0.16	0.17	—0.01	— 5.8	normal	normal	1.40	Normal blood in both limbs.
II	0.42	0.50	—0.08	—19.0	normal	normal	2.00	Saccharose and normal saline.
III	0.35	0.40	—0.05	—14.3	normal	normal	2.00	Saccharose and normal saline.
IV	0.48	0.48	0.00	0.0	normal	normal	1.50	Saccharose and normal saline.
V	0.71	0.70	+0.01	+ 1.4	partial rigor	partial rigor	2.10	Saccharose and normal saline.
VI	0.27	0.27	0.00	0.0	normal	normal	2.00	Saccharose and normal saline.
VII*	0.06	0.04	+0.02	+50.0	rigor	rigor	2.00	Starved 8 days. Strychnin. Saccharose and normal saline.
VIII	0.00	0.00	0.00	0.0	rigor	rigor	2.00	Starved 8 days. Strychnin. Glucose and normal saline.
IX	0.00	0.00	0.00	0.0	rigor	rigor	2.00	Starved 8 days. Strychnin. Saccharose and normal saline.
X	0.56	0.51	0.05	+ 9.8	normal	normal	2.00	Glucose and normal saline.
XI	0.71	0.58	+0.13	+22.8	normal	normal	2.00	Glucose and normal saline.

* In the case of Experiment VII the difference which is calculated to amount to 50.0 per cent increase is one which depends on a difference in weighing of a fraction of a milligram. We give the determination in this way in order that our results may appear in the same way as d the others. As a matter of fact, the amounts of glycogen in the two limbs were identical.

In order to test the conclusion that saccharose is not a direct glycogen former in muscle, several animals were made glycogen-free by starvation and the administration of sub-maximal doses of strychnin. Where an animal was succumbing to the poison, artificial respiration was resorted to. The animal was finally killed by pithing, and the limbs used for perfusion. In the case of saccharose no glycogen was formed (Experiments VII and IX). What is perhaps more remarkable is that in the case of glucose no glycogen was also formed (Experiment VIII).

This may be explained in one of two ways. Either the rigor induced interferes with the normal physiological processes, or the glycogen is consumed as fast as it is formed. In support of the latter view two experiments may be cited.

TABLE III.

Muscles.	Condition.	Glycogen.	Difference. Per Cent.
Right	normal	0.55	+24.4
Left	rigor	0.45	
Right	normal	0.28	+55.6
Left	rigor	0.18	

In an experiment in which both limbs were in rigor we found a complete disappearance of the glycogen from both limbs. The production of heat and the formation of carbon dioxid during rigor also goes to show that glycogen is used up during the process.[1]

On the other hand when glucose was added to the blood, there was a distinct formation of glycogen amounting in the one case to 9.8 per cent, in the second to 22.8 per cent.

SUMMARY.

Glycogen is not formed in the perfusion of muscle by blood containing saccharose. Muscles rendered free from glycogen by starvation and strychnin do not form glycogen either from glucose or from saccharose. Glucose does form glycogen in muscle. The content of glycogen in symmetrical muscles is practically alike.

[1] G. N. Stewart: *Manual of Physiology*, 4th ed., pp. 247, 587, 1900.

THE RELATION OF THE THYROID TO AUTOLYSIS, WITH A PRELIMINARY REPORT ON THE STUDY OF AUTOLYSIS BY DETERMINATIONS OF THE CHANGES IN FREEZING POINT AND ELECTRICAL CONDUCTIVITY.[1]

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(Received for publication January 14, 1907.)

In a previous publication[2] were reported the results of a study of the influence of extracts of the thyroid upon autolysis of liver tissue. These experiments were made to ascertain, if possible, the relation of autolysis to normal cellular metabolism; and they were based upon the well-known fact that administration of thyroid preparations is followed by an increased elimination of nitrogenous katabolites. If it could be found that thyroid extract exerts an augmenting effect upon autolytic decomposition of tissues *in vitro*, such a result might be considered as indicating that the autolytic enzymes are factors in proteid metabolism, and also that the thyroid modifies proteid metabolism through an effect upon the autolytic enzymes. The results of the experiments were, however, entirely negative. Liver tissue was permitted to undergo autolysis under toluol at 37° C. for periods of from 7 to 22 days, and the amount of autolysis was determined by ascertaining the proportion of the nitrogen compounds which existed in the three forms: (1) Coagulable; (2) non-coagulable, but precipitable by zinc sulphate in acid solution; and (3) that which was neither coagulable nor precipitable. It was found that the rate of autolysis of normal liver tissue of dogs was quite the same whether the tissue underwent autolysis by itself, or with the addition of various quantities of extracts of thyroid or kidney tissue.

[1] Presented before the Chicago Pathological Society, January 14, 1907.
[2] Wells: *Amer. Journ. of Physiol.*, xi, p. 351, 1904.

Later Schryver[1] reported a series of experiments by a different method, and with positive results. He fed cats with thyroid preparations for several days, and found that liver tissue obtained from such animals underwent autolysis somewhat more rapidly during the first twenty-four hours than did liver tissue obtained from control animals, which had been kept under exactly the same conditions except that no thyroid was given. However, it was found that if the animals were fed thyroid for more than seven days the results were just the opposite, autolysis being slower in the thyroid-fed animals than in the controls.

In comparing the results obtained by Schryver with those previously described, an essential difference is noted, in that Schryver studied the changes occurring during the autolysis of the first twenty-four hours, whereas in the experiments *in vitro* the amount of autolysis was not determined until several days had passed. It might well be that the difference in the results depends upon this difference in time, for it would be possible that the thyroid stimulates the rate of autolysis greatly during the first twenty-four hours, and yet after several days, when the changes are proceeding very slowly, the curve of the control autolysis might meet that of the thyroid-stimulated autolysis. To complete the studies previously reported, therefore, it was necessary to ascertain what effect, if any, thyroid extract has upon the rate of autolysis in the earliest stages.

Such a series of experiments, when properly controlled, requires the making of a very large number of nitrogen determinations, and, furthermore, requires so much material that it is often difficult to make as many experiments as desirable with material obtained from a single animal, which alone gives comparable results. Therefore determinations were made by cryoscopic methods, which permit of the making of a large number of observations in a brief space of time, and with a minimum amount of material. The details of the methods employed, and their advantages and disadvantages, will be discussed below. It may be here stated that the results indicate: First, that valuable and accurate results concerning the disintegration of proteid molecules can be obtained by means of freezing-point determinations.

[1] *Journ. of Physiol.*, xxxii, p. 159, 1905.

This can be seen by an examination of the diagrams given below; thus, Fig. 1 shows how closely together run the curves of autolysis of different specimens from the same liver, and how much more slowly autolysis occurs in muscle tissue. Secondly, they show that the addition of thyroid extract to liver or muscle emulsions has no appreciable effect upon the autolytic disintegration of cellular proteids, even during the first hours of autolysis.

THE MEASUREMENTS OF AUTOLYSIS BY CRYOSCOPY.

Determination of the rate of disintegration of proteids by means of measurements of the change in the freezing point of solutions containing them, has, so far as we can ascertain, been seldom used. Cryoscopic measurements of the osmotic pressure of living tissues seems to have been first suggested by Sabbatani[1] in connection with a study of the relation of the osmotic pressure of plant cells to their resistance to freezing, which study was carried out by Cavara. Sabbatani himself studied the freezing point of various animal tissues, his method being simply to embed the bulb of the thermometer in a piece of tissue which had been placed in a freezing mixture; the temperature was read at one minute intervals, and the freezing point was indicated by a sudden rise in the height of the mercury, after a period of supercooling, to a point where it remained stationary for several minutes before beginning to fall again. By this means fairly constant results were obtained. Neither Cavara nor Sabbatini made any studies of the effects of autolysis, although the latter noted that rapid changes in the freezing point occur rapidly after death.

Leon Frédericq[2] introduced another method of determining the freezing point of tissues. This consists of extracting the tissue thoroughly with boiling water, evaporating the extract to dryness, redissolving the residue in a quantity of water equal to the amount of fluid originally contained in the fresh tissues, and determining the freezing point of this solution. The coagulable proteids, which are left out of consideration in this method, exert no appreciable influence upon the freezing point, and therefore may be safely disregarded. (The possibility of loss of volatile

[1] *Arch. ital. de biol.*, xxxvi, p. 440, 1901.
[2] *Bull. Acad. Med. Belg.*, Seance du 29 Nov., 1902.

products of autolysis, such as volatile acids or ammonia, does not seem to have been considered.) Frédericq observed the effect of autolysis upon the freezing point, and correctly attributed it to the decomposition of large molecules of proteids and carbohydrates into smaller molecules with consequent greater effect upon the freezing point.

Delrez[1] studied the effect of autolysis upon the freezing point of muscle tissue, using generally the method of Frédericq; and Liagre[2] made a similar study of liver autolysis. In the case of muscle tissue, Delrez observed that the freezing point sometimes sank slightly after the first hour, then rose quite rapidly for about eight hours, after which the changes were slight until putrefaction set in. In aseptically preserved muscle he found the increase in the depression of the freezing point greatest during the first twenty-four hours, and relatively insignificant thereafter. Liagre found that aseptically preserved liver tissue also showed its most rapid autolysis during the first few hours after removal of the tissues from the body. Both authors proved by direct analyses that the change in freezing point corresponds to the decrease in the amount of coagulable proteid present in the tissues.

The principle involved in this method of studying the rate of autolysis is extremely simple, being briefly as follows: The effect of any substance upon the freezing point of a liquid in which it is dissolved, depends directly upon the number of molecules and ions that are in solution—the nature of the molecules and ions does not modify their effect in the least. Consequently, proteids, with their enormous molecules, have so little effect upon the freezing point that it can be practically disregarded. However, as the proteid molecules break down into their components the number of molecules in solution is greatly increased, and a corresponding effect upon the freezing point may be observed. Hence by determining the changes in the freezing point of a solution containing proteids undergoing digestion, we obtain an accurate measure of the extent and rate of this disintegration. The simplicity and rapidity of application of this method, and the small

[1] *Arch. internat. de physiol.*, i, p. 159, 1904.
[2] *Ibid.*, p. 172.

amount of material needed for making the freezing-point determinations, makes the method very advantageous for certain lines of research.

It is also possible to ascertain something as to the nature of the new-formed molecules which are increasing the depression of the freezing point, by making simultaneous determinations of the electrical conductivity of the same or similar material. The electrical conductivity of a solution depends upon the number of electrolytes present, and is not modified by the non-electrolytes. Hence, if in a given specimen the curves of the freezing point and the electrical conductivity run parallel to one another, it is evident that the new-formed molecules which are being derived from the autolyzing tissue are electrolytes, and not non-electrolytes, such as sugars and fats. In the experiments so far made on this point, we have found that the curves of freezing point depression and of electrical conductivity parallel each other very closely; indeed it is quite possible that subsequent experiments will show that the conductivity method gives quite as valuable information as to the rate and degree of autolysis as does cryoscopy, and, on account of the influence of the conversion of glycogen into sugar upon the freezing point, conductivity figures may be more useful than cryoscopic results in studying autolysis of tissue. If that is the case, the conductivity method will be found to possess greater advantages of simplicity and rapidity of determination, and to offer the possibility of making an almost indefinite number of determinations in a single small sample kept under constant conditions. This question will be the subject of further investigations.

The results which we have obtained with our experiments have been so uniform and have checked with one another so well, that we feel great confidence in the value of the cryoscopic method for detecting very slight changes in molecular concentration produced by autolytic disintegration, and we believe that it is perhaps more accurate for such purposes than is the usual method of determining the proportion of the nitrogen in coagulable and non-coagulable form. Our experiments are as yet not extensive, this paper being intended merely as a preliminary report, and it is hoped that further experience will permit of improvements on the method, and extensions of its applicability.

DETAILS OF EXPERIMENTS AND METHODS.

Dogs were used as the source of material in all the experiments. After being kept without food for twenty-four hours they were narcotized with morphine, and killed by bleeding from both carotids; the bodies were suspended by the hind feet, to remove as much blood as possible. The organs to be studied were removed as rapidly as possible, ground to a fine pulp in a hashing machine, and the pulp squeezed through cloth. Since it was desired to try the effect of thyroid extract upon autolysis, it was not possible to adopt the method of Frédericq, which possesses the advantage of permitting of studies of aseptic autolysis with perhaps less difficulty than by any other method yet proposed. All steps in our experiments were performed as rapidly as possible, so that the material was ready for the initial determination of the freezing point in less than thirty minutes after the animals had been killed.

SERIES I. Three portions of liver pulp, each weighing 50 grams, were placed in flasks, and distilled water added to make up to 500 grams. To flask No. 1 were added 25 cc. of a 10 per cent emulsion of kidney tissue from the same dog; to No. 2 were added 25 cc. of a 10 per cent emulsion of fresh sheep thyroid, which had been removed from the animals about three hours previously; to No. 3, for control, were added 25 cc. of a 10 per cent emulsion of the original liver tissue. In a second set of flasks were placed, in the same proportions as with the liver, the following mixtures: No. 4, muscle + kidney; No. 5, muscle + thyroid; No. 6, muscle + muscle. The freezing point of a 20 cc. sample of each mixture was determined at once with the usual Beckmann apparatus; the remainder was placed in stoppered flasks in the incubator at 35° C. At frequent intervals samples were taken from each flask, and the freezing point determined. The results obtained were as follows:

No. 1, Liver plus Kidney. No. 2, Liver plus Thyroid.
No. 3, Liver plus liver.

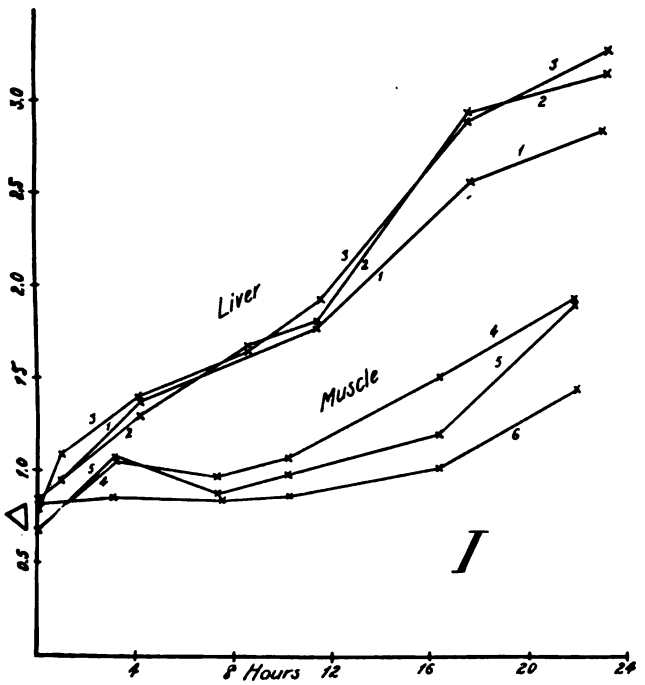
I.		II.		III.	
Time.	Δ*	Time.	Δ	Time.	Δ
12:35 p. m.	0.85	12:40 p. m.	0.86	12:50 p. m.	0.80
1:35	0.95	1:40	0.96	1:50	1.10
4:40	1.39	4:45	1.30	4:50	1.40
9:05	—	9:15	1.68	9:20	1.66
11:50	1.71	12:00	1.81	12:10 a. m.	1.93
6:00 a. m.	2.57	6:05 a. m.	2.95	6:10	2.90
11:20	2.84	11:40	3.17	11:45	3.30

* Δ = the depression of the freezing point below 0° C. multiplied by 10 on account of the dilution of the liver emulsion to $\frac{1}{10}$th strength.

No. 4, Muscle plus Kidney. No. 5, Muscle plus Thyroid.
No. 6, Muscle plus Muscle.

IV.		V.		VI.	
Time.	Δ	Time.	Δ	Time.	Δ
1:10 p. m.	0.68	1:20 p. m.	0.67	1:25 p. m.	0.82
4:20	1.06	4:25	1.08	4:30	0.86
8:30	0.97	8:40	0.89	8:50	0.85
11:20	1.08	11:30	0.99	11:40	0.87
5:30 a. m.	1.51*	5:40 a. m.	1.10	5:50 a. m.	1.01
10:50	1.94*	11:05	1.93*	11:15	1.45*

From these figures have been constructed the curves shown in Fig. I.



AUTOLYSIS OF LIVER AND MUSCLE, WITHOUT ANTISEPTICS.

1, Liver extract plus kidney extract; *2*, Liver extract plus thyroid extract; *3*, Liver extract plus liver extract (control); *4*, Muscle extract plus kidney extract; *5*, Muscle extract plus thyroid extract; *6*, Muscle extract plus muscle extract (control).

It can be seen from the results that the addition of either thyroid extract or kidney extract to an emulsion of liver tissue in

*Putrefaction,

distilled water[1] has no appreciable effect upon the rate of autolysis, as determined by the changes in the freezing point; the curves are approximately the same in each of the three sets of determinations. The relatively slight degree of autolysis of muscle tissue is also well brought out. The somewhat higher rate of autolysis seen in the specimens of muscles to which thyroid and kidney extracts have been added probably depend upon the autolysis of the thyroid and kidney extracts themselves, rather than upon any effect upon the muscle tissue. If these curves are compared with those obtained by analytic methods by Schryver for the autolysis of liver during the first sixteen hours, they are seen to agree quite closely. Certain authors have referred to a latent period lasting for the first four hours or so, but this is not shown by our results nor by the results of Schryver, at least for the liver. In the case of the muscle there is first a slight increase in the depression (Δ), followed by a distinct fall, which persists from the eighth to the twelfth hour. Delrez obtained a similar decreased depression for a brief period in one experiment, but not so frequently as with our experiments. The significance of this phenomenon is not clear, and it is perhaps not a constant one. It may be related to the observations of Galeotti,[2] who found that the electrical conductivity of cells decreases at the time of their death, which he attributes to a decrease in the degree of ionization of the cellular constituents, for the effect on depression of the freezing point was much less than the decrease in conductivity.

Series II. *The Effects of Antiseptics upon Autolysis.*

In the preceding experiments putrefaction made all results obtained after the twentieth hour of autolysis, of doubtful value. The muscles are affected by putrefaction earlier than the liver, which has been found to be the rule in other experiments. This difference between the time of putrefaction in the muscle and liver may possibly depend upon the greater autolysis of the latter, for, according to Conradi,[3] the products of autolysis exert a

[1] Salt solution cannot be used as the relatively great effect of the salt upon the freezing point would mask to a large extent the effects of the products of autolysis.

[2] *Zeitschr. f. Biol.* xlv, p. 65, 1903.

[3] *Beitr. z. chem. Physiol. u. Path.*, i, p. 193, 1901.

distinct antiseptic action. In order to control this interference by putrefactive organisms the use of antiseptics was tried. It was found by experimentation that chloroform could be used as an antiseptic with safety, as regards its modification of the freezing point of the solution, provided the water used for preparing the tissue emulsion was first saturated with chloroform at the temperature of the experiment. If the chloroform is not added until the time of the experiment, its slow solution in the water modifies the curve greatly. As has been observed in studies of autolysis by means of analytic methods, the rate of autolysis is somewhat slower in the presence of antiseptics than without them, undoubtedly because of an inhibitory effect of the antiseptics upon the enzymes. Studies of the influence of various antiseptics upon autolysis, with reference to their applicability in determinations made by physical methods, are now under way. The effects of the presence of chloroform upon autolysis may be seen in the following table and chart:

Nos. 1 and 2, Liver without chloroform.

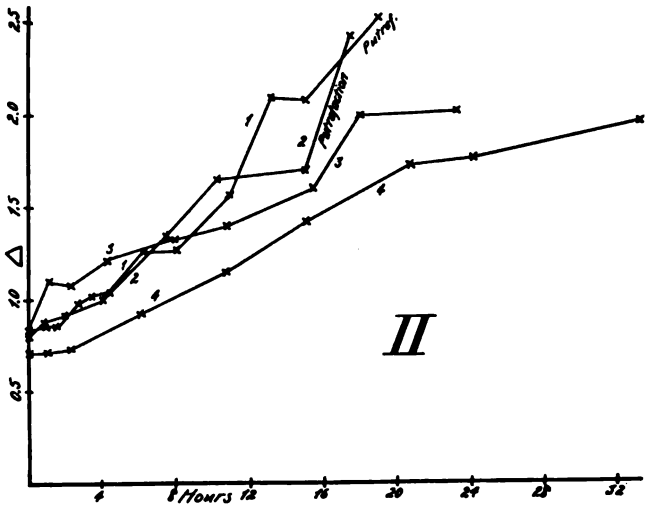
Time.	Δ	Time.	Δ
1:05 p. m.	0.84	3:10 p. m.	0.80
2:00	0.87	4:05	0.89
2:30	0.87	5:05	0.91
3:45	0.99	7:05	1.00
4:30	1.02	10:45	1.35
5:30	1.04	1:30 p. m.	1.65
7:15	1.27	6:10	1.70
9:10	1.27	8:45	2.42*
12:10 a. m.	1.57		
2:15	2.10		
4:10	2.09		
8:05	2.53*		

*Putrefaction.

Nos. 3 and 4, Liver with chloroform.

Time.	Δ	Time.	Δ
3:00 p. m.	0.83	5:00 p. m.	0.71
4:10	1.10	6:05	0.71
5:15	1.07	7:20	0.73
7:15	1.20	10:55	0.92
10:55	1.32	3:40 a. m.	1.15
1:45 a. m.	1.40	8:00	1.42
6:25	1.60	1:45 p. m.	1.73
9:00	2.00	5:10	1.77
3:15 p. m.	2.02	2:25 a. m.	1.98

Only Experiments 2 and 3 were made with material from the same liver.



AUTOLYSIS OF LIVER, SHOWING EFFECT OF ANTISEPTICS.

1 and *2*, curves of autolysis without chloroform; *3* and *4*, liver with chloroform. Only specimens *2* and *3* were from the same liver; *1* and *4* coming from different animals.

SERIES III. *The Effect of Autolysis upon Electrical Conductivity.*

As mentioned previously, it is possible to determine whether the newly added molecules in an autolyzing mixture are electrolytes or non-electrolytes, by comparing the curves of freezing point depression and of electrical conductivity. This was done with material from the same liver as No. 4 of the preceding series, the change in conductivity being determined in the usual way with the Wheatstone's bridge, and Arrhenius cell, the autolyzing mixture being kept at the same temperature (35°) as the material used for cryoscopic determinations. The freezing point and conductivity experiments were made simultaneously, and with material from the same animal. The effects of thyroid extract were also studied in this series. The results obtained were as follows:

A. Liver in chloroform water. Freezing point depression.
B. Liver in chloroform water. Conductivity increase.

A.

Time.	Δ
5:00 p. m.	0.71
6:05	0.71
7:20	0.73
10:55	0.92
3:40 a. m.	1.15
8:00	1.42
1:45 p. m.	1.73
5:10	1.77

B.

Time.	$\frac{1}{x}$ *
6:00 p. m.	0.1439
6:20	0.1525
7:10	0.1635
10:45	0.1778
4:15 a. m.	0.2030
8:30	0.2355
2:20 p. m.	0.3356
5:00	0.3587
2:00 a. m.	0.4263

* x = resistance of liver extract in Arrhenius cell; $\frac{1}{x}$ = the conductivity of the extract.

C. Liver plus Thyroid, in chloroform water. Freezing point depression.
D. Liver plus Thyroid in chloroform water. Conductivity increase.

C.

Time.	Δ
5:10 p. m.	0.70
6:10	0.69
7:35	0.74
11:00	0.82
3:45 a. m.	1.13
8:10	1.32
1:50 p. m.	1.63
5:15	1.64

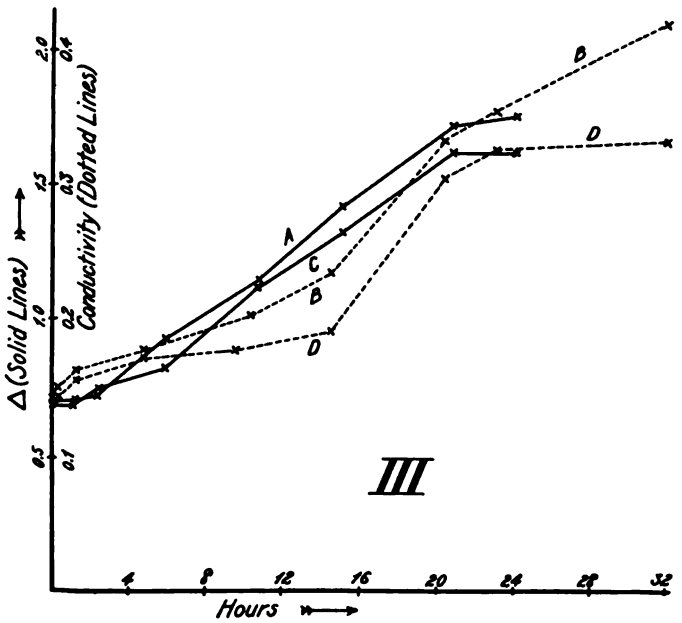
D.

Time.	$\frac{1}{x}$
6:00 p. m.	0.1398
6:20	0.1463
7:10	0.1577
10:45	0.1717
3:30 a. m.	0.1785
8:30	0.1915
2:20 p. m.	0.3065
5:00	0.3292
2:00 a. m.	0.3348

The results of this series (Fig. III) indicate again the absence of any accelerating effect upon autolysis, at least *in vitro*, by thyroid extract. Indeed, in nearly all cases the curve runs slightly lower in the specimens to which thyroid extract has been added. This probably depends upon the very slow rate of autolysis of the added thyroid extract itself, for thyroid cells undergo autolysis very slowly, a fact previously ascertained both chemically[1] and microscopically.[2] The very close parallelism of the conductivity and freezing point curves indicates that the depression of the freezing point which results from autolysis depends upon the liberation of electrolytes; these are presumably chiefly amino-acids, and perhaps also to a slight extent free fatty acids, such as

[1] Schryver: *Biochem. Journ.*, i, p. 156, 1906.
[2] Wells: *Journ. of Med. Res.*, xv, p. 159, 1906.

lactic and acetic. Bayliss[1] suggests that the inorganic salts which are held in the proteids by absorption affinity, are also liberated during proteolysis, and exert a considerable effect upon the conductivity of solutions containing proteolyzing mixtures.



AUTOLYSIS OF LIVER, AS SHOWN BY FREEZING POINT AND CONDUCTIVITY.

Freezing point, solid lines; conductivity, dotted lines. *A* and *B*, liver extract alone; *C* and *D*, liver extract plus thyroid extract.

CONCLUSIONS.

It is possible to measure the rate and degree of autolytic disintegration of tissues by determining the change in the freezing point and electrical conductivity of solutions containing the autolyzing tissues, and the products of their autolysis. This method possesses great advantages because of the ease of application and the small quantity of material required, especially in investigations in which numerous determinations are necessary for the purpose of plotting curves, etc.

[1] Quoted by Starling: *Recent Advances in the Physiology of Digestion*, p. 25, 1906.

Autolysis is much more rapid with liver tissue than with muscle tissue; but in either case it is most rapid between the twelfth and twentieth hours, at 35°.

The addition of extracts of thyroid or kidney tissue, to emulsions of liver or muscle tissue, does not greatly modify the rate of autolysis.

The effect of autolysis upon the depression of the freezing point seems to depend upon molecules which are electrolytes, and, therefore, presumably chiefly amino-acids.

THE RELATION OF EXTRACTIVE TO PROTEIN PHOSPHORUS IN ASPERGILLUS NIGER.[1]

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(Received for publication, January 23, 1907.)

The relation of the three main groups of phosphoric acid derivatives, protein, lipoid, and extractive, to one another has been the subject of considerable study. A comparison of the chemical structure of these various combinations would suggest that the more complex, like nucleins and lecithins which are colloidal in nature, are built up at the expense of simpler ones like glycerophosphoric acid and diethoxydiphosphoric acid which are soluble in water. The formation of lecithin at the expense of phosphates in the germinating seed has been demonstrated by Maxwell[2] and also by Stoklasa.[3] Their method of estimating lecithin was not strictly accurate but the differences in the amounts of lecithin found were sufficiently great to justify their conclusions. More recently Wilfarth, Römer, and Wimmer[4] have shown that a decrease in the phosphates of the straw, with a corresponding increase of protein and lecithin phosphorus in the seed, occurs during the growth of the barley to maturity. Experiments involving the transfer of material, however, from one tissue to another obviously do not permit of such definite conclusions. The same difficulty applies to the interpretation of

[1] These results are published in this preliminary form on account of changes which make it impossible for us to continue the work together.

[2] Maxwell, W.: *Amer. Chem. Journ.*, xiii.

[3] Stoklasa, J.: *Sitzungsber. d. kais. Akad. d. Wissensch. in Wien.*, Math. naturw. Klasse, civ, Ab. i, 1896.

[4] Wilfarth, Römer and Wimmer: *Landwirtsch. Versuchstat.*, lxiii, p. 1, 1905.

the results of Miescher[1] and Noel Paton[2] as to the transfer of phosphorus in the salmon during the growth of the sexual organs. There is indeed an actual loss of phosphorus from the muscle which corresponds to the gain of phosphorus in the testicle and ovary, but the relative amounts of nuclein and extractive phosphorus in the muscle are changed very little. It seemed of interest, therefore, to devise an experiment in such a way as to demonstrate a change in the relative proportion of protein to extractive phosphorus on one tissue. *Aspergillus niger* was selected for the purpose, as it is known to yield relatively good crops even with very small amounts of phosphates at its disposal. Higher plants under the same conditions would be stunted in their growth or arrested in some part of their development. Grown on the usual culture media the phosphorus of *Aspergillus niger* will be distributed about as follows, the figures expressing per cent of total phosphorus:

Protein phosphorus	29.0
Extractive phosphorus	68.0
Lecithin phosphorus	3.0

The experiments were carried on as follows:

The culture medium which represents the control solution for determining the amount of growth under favorable conditions, consisted of:

1.0 gram acid potassium phosphate.
2.0 grams ammonium nitrate.
0.5 gram magnesium sulphate.
10.0 grams cane sugar.
200 cc. distilled water.

The other solutions were made up by reducing the amount of acid potassium phosphate and keeping the other constitutents the same. The supply of potassium was kept constant by the addition of a corresponding amount of potassium nitrate to the solutions which contained less acid potassium phosphate. The solutions were placed in wide mouthed jars (Mason jars) a thick piece of cotton batting tied over the mouth and then sterilized in an autoclave at 110° C. for twenty minutes. When cool the solutions were inoculated with spores from a pure culture of *Aspergillus niger* and kept at a temperature of from 21° to 25° C. The crops were all collected

[1] Meischer, F.: *Die histochemischen und physiologischen Arbeiten*, Leipzig, 1897.

[2] Noel Paton, D.: Report of Investigation on the Life History of the Salmon in Fresh Water, Fishery Board for Scotland, p. 143, 1898.

on the same day washed, placed on filter papers to drain, dried over calcium chlorid, and weighed. For the chemical analysis the method of Koch for protein and extractive phosphorus and of Koch and Woods[1] for lecithin phosphorus was employed. As the amount of lecithin was, however, very small the figures are not given. It was found to be present in appreciable quantity in every case.

The following table gives the results of the chemical analysis:

Wt. of Crop. gm.	Wt. of KH_2PO_4 Supplied. mgm.	Composition in Per cent of Air dry Substance.			Ratio of Protein P to Extractive P.
		Protein P (nuclein).	Extractive P.	Lecithin P.	
2.410	1000	0.22	0.66	0.020	100 : 300
2.107	200	0.21	0.60	present	100 : 290
1.778	100	0.25	0.71	present	100 : 288
1.466	20	0.18	0.12	present	100 : 66
1.083	10	0.18	0.03	present	100 : 17

In the first two experiments the extractive phosphorus may have been influenced by the phosphates of any adhering culture medium. This does not apply to the last three experiments however, as there the culture medium was found to be free from phosphates at the end of the experiment.

The amount of nuclein phosphorus remains fairly constant in spite of the great decrease in the amount of phosphate supplied. The relatively good yield of crop is also interesting considering the extreme phosphorus starvation, namely, one-hundredth the usual amount. It is surprising to note to what an extreme degree the starvation (one-fiftieth) must be carried before there is any decrease in extractive phosphorus.

In conclusion the following observations seem justified:

Protein, or, in this case, *nuclein phosphorus* is, as we already know from histological evidence, the most important form of phosphorus at the disposal of the cell. It is formed at the expense of other forms (except lecithin) and is not decreased even in extreme starvation.

Lecithin phosphorus is next in order of importance. In the building up of the nucleins, however, lecithin probably takes no direct part. When lecithin is broken up in the course of its metabolism some or all of its phosphorus may be built up second-

[1] Koch, W. and Woods, H. S.: This *Journal*, i, p. 203, 1906.

arily into nuclein, merely as a matter of economy on the part of the organism.

The *extractive*, water-soluble forms of *phosphorus*, are undoubtedly the ones from which the others are built up. They would represent the intermediary step between the phosphates and the more complex combinations of phosphoric acid.

THE RELATION OF ELECTROLYTES TO LECITHIN AND KEPHALIN.

By W. KOCH.

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(Received for publication, February 4, 1907.)

In a previous study[1] of the precipitating action of calcium salts on solutions of lecithin it was found impossible to explain why sodium chloride should prevent the settling out of the precipitate. Since then the subject of the precipitation of colloids by electrolytes has been greatly advanced by the work of Mathews.[2] It seemed of interest, therefore, to again take up the subject and apply his results on albumin to colloidal solutions of lecithin and kephalin. According to Mathews the concentration of an electrolyte which is just sufficient to precipitate an electro-negative colloid bears some relation to the solution tension of its ions. The greater the solution tension of the kation the greater must be its concentration necessary to cause precipitation. The anion in such a case exerts a dissolving influence which also bears a relation to its solution tension. Sodium chloride would, therefore, prevent the precipitation of lecithin by calcium chloride on account of the dissolving action of the chlorine ion.

The experiments were carried on with a sample of A. G. F. A. lecithin made from eggs. The sample had been previously analyzed by Woods[3] and found to consist of two-thirds kephalin. The solution was made up by shaking 1.0 gram of lecithin with 500 cc. of distilled water in a shaking machine for several hours. This insured a perfectly fresh solution which was then filtered before using. The tests were all made in test-tubes of 20 cc. capacity. A sufficient amount of the salt was measured out

[1] Koch, W.: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 181, 1903; *The Decennial Publications of the University of Chicago*, x, p. 93, 1902.

[2] Mathews, A. P.: *Amer. Journ. of Physiol.*, xiv, p. 203, 1905.

[3] Koch, W. and Woods, H. S.: This *Journal*, i, p. 211, 1906.

from a standardized solution to bring the concentration up to the one given in the table when diluted to 10 cc. Five cc. of lecithin solution were used in every case and the total made up to 10 cc. The following is an illustration: 1.7 cc. $\frac{M}{50}$ $CaCl_2$ + 3.3 cc. H_2O + 5 cc. of lecithin solution gave 10 cc. of solution in which the concentration of the calcium would be 0.00345M. In order to decide if precipitation had taken place or not the tube was allowed to stand twenty hours. This forms a convenient time as a solution which will not cause the lecithin to settle out in twenty hours will not bring this about if allowed to stand several days longer, although the solution may be more turbid than the control. The following table gives the results.

TABLE I.

Concentration of Kation Required to Precipitate.		Concentration of Anion Required to Prevent Precipitation.				
KCl	0.3 M	NaOH	0.0066 M	prevents ppt. by	0.33 M	NaCl
NaCl	0.15 M	NaOH	0.0066 M	does *not* prevent ppt. by	0.005 M	$CaCl_2$
$SrCl_2$	0.006 M	NaOH	0.01 M	prevents ppt. by	0.005 M	$CaCl_2$
$MgSO_4$	0.004 M	NaI	0.1 M	prevents ppt. by	0.005 M	$CaCl_2$
$CaSO_4$	0.0033 M	NaBr	0.2 M	does *not* prevent ppt. by	0.005 M	$CaCl_2$
$CaCl_2$	0.0030 M	NaBr	0.05 M	prevents ppt. by	0.0033 M	$CaCl_2$
H_2SO_4	0.002 M	NaCl	0.1 M	does *not* prevent ppt. by	0.0033 M	$CaCl_2$
$CuSO_4$	0.00125 M	NaCl	0.33 M	+ NaOH 0.0066 M prevents ppt. by 0.01 M $CaCl_2$		

The arrangement of salts in the above table is practically the same as that found by Mathews for colloidal albumin solutions. The sensitiveness of lecithin to this precipitation is so great that it is possible to distinguish between two solutions which differ from one another by only 0.1 mg. of calcium in 10 cc.

If we measure the changes in viscosity of the solution the reaction becomes even more delicate. The addition of an amount of calcium chloride not sufficient to precipitate the lecithin in twenty-four hours will cause a decrease in the viscosity of the solution. The measurements were made with an Ostwald viscosimeter and represent rate of flow through a capillary tube.

1. For distilled water ..... 57 seconds.
2. For emulsion of lecithin ..... 65 seconds.
3. +0.00125M $CaCl_2$ ..... 63 seconds.
4. +0.0025 M $CaCl_2$ ..... 60 seconds.
5. +0.0033 M $CaCl_2$ ..... 57 seconds.
6. +0.005 M NaOH ..... 67 seconds.

From the above it will be seen that as soon as the concentration of the calcium becomes sufficient to cause a precipitation of the lecithin (5) the viscosity becomes equal to that of distilled water. Sodium hydrate has the opposite effect, increasing the viscosity.

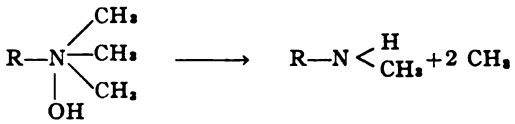
The previous observation[1] made on lecithin from sheep's brains to the effect that sodium and potassium chlorides do not precipitate lecithin are apparently not confirmed by the results given in the first column of Table I. A repetition of these experiments with crude preparations of lecithin from human brains revealed the curious result that a preparation from corpus callosum behaved like egg lecithin while a preparation from cortical gray matter behaved like the lecithin previously obtained from sheep's brains. This at first suggested that the lecithin from gray matter might be different from that made from eggs or corpus callosum. The fact that a concentration of 0.0066M sodium hydrate prevents the precipitation of egg lecithin by sodium chloride and moreover makes it possible for the sodium chloride to prevent precipitation by calcium (see second column of Table I) suggests the possibility that the lecithin of the cortex may be in combination possibly as an ion-lecithin compound. Another explanation was, however, found to be correct. Egg lecithin and lecithin from corpus callosum are very rich in kephalin. When the crude lecithin from corpus callosum was freed from kephalin by dissolving in cold alcohol (which leaves most of the kephalin behind insoluble in this case) a preparation was obtained which more nearly resembles lecithin from the cortex. The following table gives the results:

TABLE II.

Crude Lecithin from	Concentration Required to Precipitate Calcium Chloride.	Sodium Chloride.
Eggs (⅔ kephalin)	0.00300 M	0.16 M
Corpus callosum (½ kephalin)	0.00345 M	0.20 M
Cortical gray matter (⅛ kephalin)	0.00588 M	2.0 M (no ppt.)
Corpus callosum (purified)	0.0059 M	1.0 M

[1] Koch, W.: *Loc. cit.*, p. 183.

The greater sensitiveness of kephalin to precipitation by kations can be explained by its more acid properties. It is well known that the addition of a slight amount of acid to an electro-negative colloid greatly increases the ease with which it is precipitated. Kephalin among other things differs from lecithin by the absence of two methyl groups, and the consequent change of the tetra ammomium base of the cholin to a triad nitrogen is sufficient to explain the decrease in basic properties.



The fact that preparations representing mixtures of lecithin and kephalin from corpus callosum are precipitated by 0.2 M or about one per cent of sodium chloride suggests an interesting explanation of Macallum's[1] observation, namely, that sodium chloride (which is most probably the chloride present) is confined to the axis cylinder of a nerve fiber and does not penetrate the medullated sheath. The medullary sheath contains large amounts of kephalin as well as cholesterin and cerebrin (in per cent of dry matter of corpus callosum there are: Kephalin, 14 per cent; cholesterin, 15 per cent; cerebrin, 17 per cent; lecithin, 14 per cent). Such a mixture would be extremely sensitive to precipitation by sodium chloride and would consequently form in contact with sodium chloride solution a precipitation membrane similar to that formed by a drop of copper sulphate, which has been suspended in a potassium ferrocyanide solution. The sodium chloride cannot diffuse through such a membrane any more than the copper sulphate diffuses through the semipermeable membrane of copper ferrocyanide. The cholesterin no doubt plays a rôle in rendering the kephalin more sensitive to precipitation and in strengthening the membrane when formed. The starch-like cerebrin acts as a filler and the neurokeratin network gives further stability to the medullary sheath.

The application of these results to the red blood cells, which are also quite rich in kephalin and cholesterin, seems very promising and will be continued.

[1] Macallum: *Proc. of the Roy. Soc.*, lxxvii, Series B, p. 165, 1906.

EXPERIMENTS BEARING UPON THE MODE OF OXIDATION OF SIMPLE ALIPHATIC SUBSTANCES IN THE ANIMAL ORGANISM

(ACETIC ACID, GLYCOLLIC ACID, GLYOXYLIC ACID, OXALIC ACID, GLYCOCOLL AND GLYCOL.)

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The study of the mechanisms of the chemical oxidations carried out in the animal body is a problem which has long been recognized to be of great importance, but the prosecution of this line of research has met with so many experimental difficulties that at the present time our knowledge of the subject is extremely meagre. It is not yet possible to form a complete picture of the mechanism of the oxidation of simple aliphatic substances containing two or three carbon atoms; in fact, more is known of the fate of complex substances belonging to the aromatic series than those of the aliphatic group.

Oxidation of saturated substances, both by direct chemical means and through the agency of the living organism usually consists in the replacement of hydrogen atoms by hydroxyl groups with formation of products which may or may not undergo further decomposition. In the case of oxidations carried out by the organism, the primary products are so seldom capable of resisting further change that the intermediate stages between the primary and end products of oxidation are usually unknown. The possibility of actually isolating intermediate products of oxidation from animal tissues and excretions is in a large measure dependent upon the stability of the intermediate products and whether the rate of their formation is great compared with the rate of their decomposition. Unfortunately these conditions seldom prevail.

By far the most satisfactory experimental method for the investigation of the course of oxidations would be to submit

single, definite compounds, such as are normally dealt with by the living organism, to the action of roughly purified oxidizing enzymes. Unfortunately we have no grounds for believing that the oxidizing enzymes (oxygenases, peroxidases, aldehydases, etc.) that have been studied apart from living cells, have anything to do with the *main* changes of catabolism, since they are without action upon the chief groups of substances which furnish energy to the organism.

In some cases, in which direct chemical methods have been inapplicable, indirect pharmacological methods have proved of service; an example of this kind is found in the demonstration of the formation of glycuronic acid as an intermediate product of the oxidation of glucose and its excretion in the urine in combination with drugs, such as aromatic alcohols.

A more direct method for the detection of intermediate products of oxidation is that employed by Paul Mayer[1] and Hildebrandt,[2] and others, who have administered to animals enormous doses of substances normally present in but small quantity. When sufficiently large doses are employed, part of the substance escapes oxidation and appears in the urine. In this way it is possible to so fully utilize the oxidative capacity of the organism that in some cases intermediate products of oxidation are protected from further attack and so are excreted and may be detected in the urine. In this way it has been shown that oxalic acid appears in the urine when an alimentary glycosuria is set up in rabbits by the administration of large quantities (forty-five grams) of sugar.

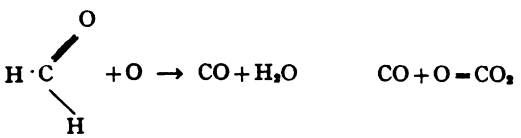
Both the pharmacological method of investigation and that involving the administration of very large quantities of substances must be regarded as very unphysiological, and in fact some writers go so far as to ascribe no value to results obtained by these methods. Although I think it can hardly be doubted that very valuable results have been and may yet be obtained by following these lines of investigation, there is another method which, though beset with very real drawbacks, does in some respects involve less departure from the normal course of events

[1] Paul Mayer: *Deutsch. med. Wochenschr.*, 16–17, 1901; *Zeitschr. f. klin. Med.*, vlxii, p. 68, 1902; *Zeitschr. f. physiol. Chem.*, xxxviii, p. 135, 1903.

[2] Hildebrandt: *Ibid.*, xxxv, p. 141, 1902.

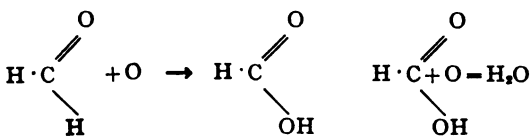
occurring in the organism. This mode of investigation of the intermediate products of oxidation of a substance consists first of all in a theoretical consideration of the different products which may be formed, followed by examination of the separate behavior of each of these products in the organism. In some cases, at least, the results will be such as will give a clear indication of some of the steps in the course of the degradation of the substance.

The simplest example of the application of this principle may be seen in the determination of the fate of formaldehyde in the organism. When only catabolic changes are considered it will be seen that the substance may theoretically be oxidized in the following ways:



$$H\cdot C(=O)H + O \rightarrow CO + H_2O \qquad CO + O = CO_2$$

or



$$H\cdot C(=O)H + O \rightarrow H\cdot C(=O)OH \qquad H\cdot C(=O)OH + O = H_2O + CO_2$$

That is to say, that although the final products in each case are carbon dioxide and water, the intermediate products may be either carbonic oxide or formic acid. Further experiment shows that carbonic oxide is not only poisonous but is absolutely resistant against further oxidation by the organism. On the other hand it is found that formic acid is actually oxidized to carbon dioxide in the organism. It is, therefore, concluded that formaldehyde is oxidized in accordance with the second equation and this is confirmed by the fact that the normal formic acid excretion in the urine is increased after consumption of formaldehyde derivatives. The application of considerations based on the assumption that certain substances are attacked with difficulty in the organism and so cannot physiologically be considered as precursors of carbon dioxide, has been made by Pohl,[1] to several cases of oxidation.

[1] Pohl: *Arch. f. exper. Pathol.*, xxxvii, p. 413, 1895–1896.

The main objection to the method under consideration lies in the crudity of our methods of administration which are often incapable of bringing a substance in suitable quantity to the proper sphere of activity of the cells concerned in its catabolism. It is probably this difficulty which accounts for the fact that certain substances which are believed to undergo active metabolism in the body are excreted unchanged when introduced from without.

In many ways a more than superficial resemblance appears to exist between combustion taking place in flame and in the living organism, and it is not impossible that the changes by which energy is liberated from a straight-chain paraffin and a saturated fatty acid have something in common. Our knowledge of the mechanism of the combustion of hybrocarbons is largely due to the brilliant series of researches carried out by Bone and his co-workers.[1] It has been shown that the oxidation of a hydrocarbon by both slow and explosive combustion may be regarded as a process involving the initial formation of unstable hydroxylated molecules which subsequently undergo decomposition into simpler products. For example, formaldehyde and steam may be identified at an early stage in the combustion of methane, the formaldehyde decomposing at the high temperature into carbonic oxide and hydrogen which are then burnt to carbon dioxide and water. The changes are believed to take place as follows:

$$CH_4 \rightarrow CH_3(OH) \rightarrow CH_2(OH)_2 \rightarrow H_2O + CH_2O \rightarrow CO + H_2 \rightarrow CO_2 + H_2O$$

In a similar way aldehydes are formed by the combustion of ethane and propane and undergo further decomposition at high temperatures.

The detection with certainty of these intermediate products of a reaction which usually results simply in the production of carbon dioxide and water is of great interest and may be considered as justification for the belief in the formation of substances as intermediary products of metabolism which have but a transient existence and which cannot be isolated at least

[1] W. A. Bone: *Trans. Chem. Soc.*, lxxxi, p. 536, 1902; lxxxiii, p. 1074, 1903; lxxxv, pp. 693 and 1637, 1904; lxxxvii, p. 1232, 1905; lxxxix, pp. 652 and 660, 1906.

from an organism under physiological conditions. Although the analogy between combustion in the living organism and in flame must not be pushed too far, for some of the changes in the latter, especially those due to thermal conditions, such as the breaking down of formaldehyde into carbonic oxide and hydrogen would not be likely to occur in the cell, yet it can be hardly doubted that a careful comparison of the two kinds of phenomena would be of interest.

But few experiments have been made upon the fate of the simple acids when introduced into the body. Schotten[1] showed that large quantities of the sodium salts of the saturated fatty acids could be readily burnt in the organism. Ten to twenty grams of the fatty acids from formic to caproic acid, with the exception of propionic acid, were given to dogs by mouth in the form of sodium salts. It was found that the quantity of volatile acids in the urine after the administration of caproic, valeric and butyric acids was not materially increased, indicating practically complete combustion. In the case of acetic and formic acids the results were similar except that the combustion was less complete. After administration of twenty-five grams of sodium acetate, rather less than ten per cent was recovered from the urine while after twenty grams of sodium formate about twenty-six per cent was recovered unchanged in the urine. In all cases the urines contained much sodium carbonate. Later experiments by Pohl,[2] Gréhaut and Quinquand, and by Pellacani, in the main confirm the earlier results of Schotten and tend to prove that the formates are practically completely burnt in small doses but when larger quantities are administered a part is excreted unchanged. It is perhaps not out of place to mention that these experiments in themselves are not sufficient to warrant the deduction which is commonly made to the effect that the lower fatty acids are much more difficultly oxidized than their homologues, unless the relative permeability of the kidney for the different salts is considered at the same time.

The mechanism of the combustion of formic acid does not present many possibilities and it is probably correct to assume that

[1] Schotten: *Zeitschr. f. physiol. Chem.*, vii, p. 375. 1883
[2] Pohl: *Loc. cit.*

a direct conversion into carbon dioxide and water takes place, but in the case of acetic acid several modes of combustion are theoretically possible. It is natural to suppose that the methyl group is the seat of attack and if we assume that combustion in the tissues like the combustion in flame is essentially a process of hydroxylation we may anticipate the initial formation of glycollic acid. The introduction of a second hydroxyl group would yield dioxyacetic acid which would give glyoxylic acid and water. The glyoxylic acid on further oxidation might be expected to give oxalic acid which in turn would yield carbon dioxide and water. The change may be represented as follows:

$$CH_3.COOH \rightarrow CH_2OH.COOH \rightarrow CH(OH)_2.COOH \rightarrow CHO.COOH$$
$$(H_2O) \rightarrow COOH.COOH \rightarrow 2\ CO_2 + H_2O$$

The likelihood of these reactions is increased by the fact that acetic acid and glycollic acid both yield glyoxylic acid in direct oxidation with hydrogen peroxide and glyoxylic acid in turn is readily oxidized to oxalic acid.

On the other hand the possibility must be considered of the glycollic acid decomposing in other ways. A direct decomposition into formic acid and formaldehyde is conceivable although improbable. Since, however, glycollic acid yields formaldehyde, formic acid and carbon dioxide on oxidation with hydrogen peroxide[1] a similar decomposition might take place in the organism. The aldehyde would then be oxidized to formic acid which would ultimately give carbon dioxide and water:

$$CH_3.COOH \rightarrow CH_2OH.COOH \rightarrow CH_2O.;(CO_2 + H_2O) \rightarrow H.COOH$$
$$\rightarrow CO_2 + H_2O.$$

Accordingly the possible intermediate products of the oxidation of acetic acid which we have to consider are glycollic acid, glyoxylic acid, oxalic acid, formaldehyde and formic acid. Reference must here be made to the experiments conducted by Pohl, with the object of elucidating the mechanism of oxidation of acetic acid, especially as my results in most respects do not tend to confirm this investigator's conclusions. The possibility of the reaction involving the formation of formic or oxalic acids has been rejected by Pohl on the ground that formic acid and

[1]H. D. Dakin: This *Journal*, i, p. 271, 1906.

oxalic acid do not appear in increased amounts in the urine after administration of acetates, but this does not appear to me to be altogether convincing proof. Pohl regarded oxalic acid as stable against further oxidation by the organism and so any oxalic acid found as a product of oxidation of acetic acid should appear in the urine. This, however, can be no longer regarded as correct.

Pohl pictured the oxidation of acetic acid by the organism as taking place in the stages represented by the following equations:

$$CH_3.COOH + O = CH_2.OH.COOH$$
$$CH_2OH.COOH + O = CH(OH)_2.COOH = COH.COOH + H_2O$$
$$COH.COOH + O = 2\ CO_2 + H_2O$$

The basis for this view is the fact that administration of acetates is not followed by oxaluria and Pohl believed from the results of his experiments that glycollic and glyoxylic acids were burnt in the organism without the intermediate formation of oxalic acid. New experiments by the writer have shown, however, that considerable amounts of oxalic acid are formed by the oxidation of glycollic and glyoxylic acids in the body.

It appears that at present we have insufficient evidence to picture completely the mechanism of the oxidation of acetic acid, but it is not improbable that glycollic, glyoxylic and oxalic acids are intermediate products since they are all capable of further oxidation by the organism. It must be admitted, however, that it has not been possible to induce oxaluria as the result of acetate administration (Experimental Part 1). The intermediate production of formic acid may also have to be considered, since the fact that an increased formate excretion has not hitherto been found to follow acetate administration, cannot be held to exclude this possibility.

From the fact that glycollic acid on oxidation with hydrogen peroxide yields glyoxylic acid, formaldehyde, and formic acid, one might anticipate that these products would be formed in the course of its oxidation by the organism. Since glyoxylic acid, as will be shown later, is at least in part, oxidized by the organism to oxalic acid, the presence of the latter substance might also be expected.

$$CH_2.OH.COOH \rightarrow COH.COOH \rightarrow COOH.COOH \rightarrow 2CO_2 + H_2O$$
$$CH_2.OH.COOH \rightarrow H.COH + (CO_2 + H_2O) \rightarrow H.COOH \rightarrow CO_2 + H_2O$$

Experiments (Experimental Part 2) showed that no increase in formic or other volatile acid in the urine followed the administration of glycollic acid or its salts given either by mouth or subcutaneously to rabbits and dogs; on the other hand, very definite evidence of the formation of oxalic acid was obtained, especially when the glycollic acid was given subcutaneously. In some cases the oxalic acid excretion is relatively large but under certain conditions it may be but slight and when small doses are given by mouth to dogs no increase in oxalic acid may be apparent. These results are easily explained on the grounds that the oxalic acid formed undergoes further oxidation to carbon dioxide and water. Special experiments (4) showed that moderate quantities of oxalic acid given subcutaneously to rabbits are readily burned. These results are in accord with those of Hildebrandt[1] and Bakhoven[2] (see p. 67).

When moderately large doses of free glycollic acid are given by mouth a part of the acid appears unchanged in the urine and the proportion of the total nitrogen of the urine in the form of ammonia is raised.

Since the oxidation of glycollic acid by the organism proceeds at least in part through the stage of oxalic acid, the intermediate formation of glyoxylic acid is to be inferred. As glyoxylic acid has been stated by Eppinger to form allantoin in the body it might be conjectured that an increased allantoin excretion would follow the administration of glycollic acid or its salts. In no case, where the free acid or the sodium, ammonium, or calcium salts were employed could such a change be satisfactorily demonstrated.

The combined results may be taken to show that glycollic acid is oxidized in the organism through the stages of glyoxylic and oxalic acids:

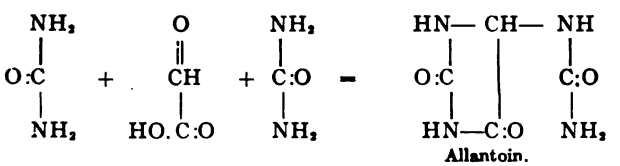
$$\begin{array}{c} CH_2.OH \\ | \\ COOH \end{array} \rightarrow \begin{array}{c} COH \\ | \\ COOH \end{array} \rightarrow \begin{array}{c} COOH \\ | \\ COOH \end{array} \rightarrow 2\ CO_2 + H_2O$$

The amounts of oxalic acid found in the urine under certain conditions indicate that a very large part, if not all, of the glycollic acid is broken down in this way, for a large part of the oxalic

[1] Hildebrandt: *Zeitschr. f. physiol. Chem.*, xxxv, p. 141, 1902.
[2] Bakhoven: *Jahresber. f. Thierchemie*, xxxii, p. 362, 1902.

acid initially formed must be completely burnt to carbon dioxide and water. No evidence was obtained tending to favor the belief that formic acid is an intermediate product in the reaction but the probability of the further oxidation of the formic acid renders negative insufficient evidence to disprove the possibility of such a change.

The fate of glyoxylic acid in the organism has been investigated with different results by Pohl and by Eppinger. The former investigator concluded from his experiments that glyoxylic acid in moderately large doses[1] was completely burnt in its passage through the organism, after the administration of sodium glyoxylate by mouth. No glyoxylic acid could be detected in the urine and oxalic acid was not considered to be a product of its oxidation, although an inspection of Pohl's analytical results reveals the presence of a distinct oxaluria. No toxic effects were noted. Eppinger, on the other hand, gave calcium glyoxylate by mouth to dogs and believed that an increased oxalic acid and allantoin excretion resulted therefrom. The formation of the latter substance would be of considerable interest as furnishing an example of the synthesis from a simple acid derivable from both proteid and carbohydrates of a substance closely allied to the purin bases. The formation of allantoin from glyoxylic acid necessitates the addition of two molecules of urea:



Eppinger's dogs which had received the glyoxylates died after some days but no definite lesions were detected.

My own experiments have been made with the free acid, with the calcium, ammonium, ands odium salts and with the methyl ester. These substances have been given to dogs and rabbits both by the mouth and subcutaneously. The results have shown that glyoxylic acid is, at any rate in part, oxidized to oxalic acid in the body, and that the proportion of oxalic acid appearing in

[1] Two grams of the free acid for small dogs (6 kilos).

the urine varies according to the mode of administration, being least when the glyoxylic acid is given by mouth. Considerable variations in the oxalic acid excretion are found in even the same animal with identical doses and this is to be explained on the assumption that the further oxidation of the oxalic acid to carbon dioxide and water is more complete under some conditions of the organism than under others. No indication was obtained of the presence of glyoxylic acid in the urine after administration of the free acid and this result is confirmed by the fact that the ratio of the ammonia to the total nitrogen of the urine is not increased. No evidence was obtained of an increased formic acid excretion although for reasons already mentioned this cannot be taken to preclude the possibility of a part of the glyoxylic acid breaking down with the production of formic acid as an intermediate product.

With regard to the formation of allantoin I am unable to confirm the results of Eppinger. An investigation of the available methods for the estimation of allantoin[1] shows that many substances other than allantoin are precipitated and estimated along with that substance, and although the "allantoin nitrogen" of the urine is frequently increased by glyoxylic acid administration, it has not been possible to isolate the allantoin in the pure state in more than the traces which are normally obtainable from urines. In the experimental portion of this paper a criticism will be found of Loewi's method for the estimation of allantoin, which was that employed by Eppinger. Both glyoxylic acid and its salts in moderate quantities[2] were found to be comparatively non-toxic, certainly less so than oxalic acid. This result agrees with Pohl's experience and is in opposition to that of Eppinger. It is perhaps conceivable that this divergence of results may be due to the possible presence of the extremely poisonous glyoxal in Eppinger's preparation.

In the view provisionally put forward that glycollic, glyoxylic and oxalic acids are intermediate products of the oxidation of

[1] Loewi: *Arch. f. exper. Path. u. Pharm.*, xliv, p. 19, 1900; Swain: *Amer. Journ. of Physiol.*, vi, p. 38, 1902; Poduschka: *Arch. f. exper. Path. u. Pharm.*, xliv, p. 59, 1900.

[2] Two grams of the acid in the form of calcium or sodium salts do not produce toxic symptoms in rabbits.

acetic acid is accepted, it is obviously necessary to have definite information as to the powers of the organism to carry the oxidation of the oxalic acid further, to carbon dioxide and water. Very many investigations have been made to decide this point but with widely differing results. According to Gaglio, Wiener and others[1] oxalic acid given subcutaneously to dogs is excreted unchanged, and the same result was obtained by Pohl when the acid was introduced into a dog with a Thiry-Vella fistula. Many experiments have been made in which destruction of oxalic acid was observed when the acid was taken by mouth,[2] but as there appears to be evidence that oxalic acid is decomposed by putrefactive agencies these experiments are not completely convincing.[3]

More modern experiments by Hildebrandt[4] upon the fate of oxalic acid injected subcutaneously into rabbits showed that from 60 to 90 per cent of the acid was oxidized in the body and experiments of my own (Experimental Part 4) have completely confirmed these results. Confirmation of these results is afforded by the fact that oxaluric acid, parabanic acid, and alloxan are completely oxidized in the body when given to dogs subcutaneously.[5] The wide differences between the older and more recent experiments can, I think, be accounted for by the use of the quite inadequate method for the estimation of oxalic acid devised by Neubauer and which for long was the only available one. In the experimental portion of this paper an account is given of the method of oxalic acid estimation employed in my own experiments.

The result of these experiments upon the fate of glycollic, glyoxylic and oxalic acids in the organism, showing that they are

[1] Gaglio: *Arch. f. exper. Path. u. Pharm.*, xxii, p. 246, 1887; Pohl: *ibid.*, xxxvii, p. 415, 1895–1896; Faust: *ibid.*, xliv, p. 236, 1900; Abeles: *Wien. klin. Wochenschr.*, Nos. 19 and 20, 1892.

[2] Baldwin: *Jour. of Exper. Med.*, v, p. 27, 1900; Marfori: *Jahresber. f. Thierchemie*, xx, p. 70, 1890; xxvi, p. 74, 1896; Lommel: *Deutsch. Archiv f. klin. Med.*, lxiii, p. 599, 1900; Barth and Autenreith: *Zeitschr. f. physiol. Chem.*, xxxv, p. 327, 1902.

[3] Stradomsky: *Virchow's Arch. f. path. Anat. u. Physiol.*, clxiii, p. 404, 1901.

[4] Hildebrandt: *Zeitschr. f. physiol. Chem.*, xxxv, p. 141, 1902.

[5] Luzatto: *ibid.*, xxxvii, p. 225, 1902; Koehne: Inaug. Diss., Rostock, 1894.

capable of oxidation and that the oxalic acid is a product of oxidation of glycollic and glyoxylic acids in the organism, appear to me to give some degree of probability to the view that the oxidation of acetic acid, at least in part, takes place with formation of these substances as intermediate products:

$$CH_3.COOH \rightarrow CH_2OH.COOH \rightarrow CH(OH)_2.COOH \rightarrow CHO.COOH + H_2O \rightarrow COOH.COOH \rightarrow 2CO_2 + H_2O$$

The question as to whether formaldehyde or formic acid are also intermediate products cannot be regarded as settled.

It might be urged that the formation of more than traces of a moderately toxic substance, such as oxalic acid as a product of normal metabolism would be unlikely; but this objection can, I think, hardly have much weight, for it is not necessary to assume that intermediate products have more than a transient existence. The final proof of the formation of formaldehyde in the assimilation of carbon dioxide by plants, furnished by the masterly experiments of Priestley and Usher, is an example of the production by the cell of an extremely powerful protoplasmic poison in the course of its normal activities. It seems reasonable to assume that in cases such as these the cell's defense consists in the presence of enzymes capable of rapidly converting the toxic substance into other products so that the concentration of the former is always infinitely small. It is also probable that the oxidation of intermediary substances formed in this way by intracellular enzymes may be much more readily accomplished than when the same substance is introduced from without in comparatively high concentration.

The steps in the progressive oxidation of substances may in some cases be so rapid that one may prefer to assume that the intermediate products have no separate existence but that the molecule of the substance undergoing change is altered in such a way that if the reaction could be stopped at that particular point, an intermediate product would be formed as the result of intramolecular rearrangement, but that normally the reaction is carried beyond this stage. This, however, is at present at any rate as regards reactions in the living tissues, of theoretical rather than practical interest.

The demonstration of the formation of oxalic acid from the

oxidation of glycollic acid in the organism is of interest in clearing up the mechanism of the oxidation of glycol. Pohl and later Paul Mayer found that injections of glycol into rabbits produced a marked oxaluria and this result has been confirmed by my own experiments (Experimental Part 5). Mayer also found that when large doses were given, glycollic acid appeared in the urine and was identified in the form of its phenylhydrazid. This result appeared somewhat anomalous in view of the statement of Pohl's that glycollic acid did not yield oxalic acid in the organism. Pohl explained the formation of oxalic acid as arising from the simultaneous oxidation of both alcohol groups, but the new experiments make it appear more probable that glycol breaks down in accordance with the following scheme:

$$\begin{array}{l} CH_2OH \\ | \\ CH_2OH \end{array} \rightarrow \begin{array}{l} CHO \\ | \\ CH_2OH \end{array} \rightarrow \begin{array}{l} COOH \\ | \\ CH_2OH \end{array} \rightarrow \begin{array}{l} COOH \\ | \\ C=(OH)_2 \\ \quad | \\ \quad H \end{array} \rightarrow \begin{array}{l} COOH \\ | \\ C=O \\ \quad | \\ \quad H \end{array} \rightarrow \begin{array}{l} COOH \\ | \\ COOH \end{array} \rightarrow 2CO_2+H_2O$$

Since glycocoll gives rise to glyoxylic acid on oxidation with hydrogen peroxide, it might be thought that glycocoll would be a source of glyoxylic acid and hence of oxalic acid in the organism. Since glycocoll is also attacked with the liberation of ammonia by many tissue ferments, the possibility of the formation of glycollic acid and of its oxidation products must be considered. At present, however, I have not been able to satisfactorily demonstrate a marked oxaluria as the result of glycocoll catabolism (Experimental Part 6). The probability that glycocoll does break down to some extent in the way outlined is strengthened when the fact is considered that gelatin, which contains large quantities of glycocoll, when consumed in large quantities does lead to a slight oxaluria in man and sometimes in dogs, although the increase in oxalic acid excretion is usually only five to fifteen milligrams. This observation was first made by Lommel[1] and has been confirmed by Stradomsky,[2] Mohr and Salomon,[3] and by the writer (Experimental Part 7).

[1] Lommel: *Loc. cit.*

[2] Stradomsky: *Loc. cit.*

[3] Mohr and Salomon: *Deutsches Arch. f. klin. Med.*, lxx, p. 486, 1901.

It may perhaps be not out of place to refer to a remarkable paper by Klemperer and Tritschler[1] in which a single experiment is described relative to the production of oxaluria in dogs as the result of glycocoll injection. These authors conclude that an increased oxalic acid excretion, equivalent to one hundred and fifty-six milligrams of oxalic acid, resulted from the injection of five-tenths of a gram of glycocoll. Such a result is astonishing, but when it is seen that the supposed oxaluria was at its maximum twenty-five days after injection, it may be inferred that the experiment is of questionable value. The same authors describe a blood ferment causing a destruction of oxalic acid, which, from the authors' figures, appears to be most active at the boiling point of the oxalic acid solution. Many other criticisms of this work may be made but they would serve no useful purpose.

Another source of glyoxylic acid and hence of oxalic acid may be sought in creatin and creatinin, which readily yield glyoxylic acid on oxidation with hydrogen peroxide. The probability of creatin being a precursor of oxalic acid was long ago realized by Kühne and recently Klemperer and Tritschler have claimed to have produced such an oxaluria experimentally. Stradomsky, however, observed only negative results and it appears that even if oxalic acid were an intermediate product of oxidation of creatin it is improbable that any appreciable amount would escape further oxidation.

Much has been written upon the source of the traces of oxalic acid which normally appear in the urine but there is but little unanimity of opinion upon the subject. The fact that oxaluria may persist after long starvation shows that the oxalic acid is not exclusively of alimentary origin. Wesley Mills[2] found that dogs excreted most oxalic acid upon a flesh diet while Haas[3] found in some cases an increase of oxalic acid followed increased carbohydrate consumption. Lüthje[4] and Stradomsky on the other hand found that the oxalic acid output was highest upon a

[1] Klemperer and Tritschler: *Zeitschr. f. klin. Med.*, xliv, p. 337, 1902.

[2] Wesley Mills: *Virchow's Arch. f. path. Anat. u. Physiol.*, xcix, p. 305, 1885.

[3] Haas: "Ueber Oxalurie," Inaugural Dissertation, Bonn, 1894.

[4] Lüthje: *Zeitschr. f. klin. Med.*, xxxv, 1898.

flesh diet, lowest on a carbohydrate-rich diet, and an intermediate result was obtained with diets containing an abundance of fat. A careful consideration of what is known of the breakdown of fats and higher fatty acids in the animal body, especially with regard to the production of β-oxybutyric acid as an intermediate product of normal metabolism does not exclude the possibility of the production of oxalic acid as a further intermediate product of oxidation. Since, moreover, there is good evidence for the possibility of its formation from carbohydrates and from some of the constituents of flesh, such as glycocoll, creatin, creatinin, and probably other substances, it appears unsafe to attempt to refer normal oxalic acid excretion to one particular class of food-stuffs. It is more probable that it may be formed from all classes of food, but that under conditions of normal metabolism only a minute fraction of it escapes further oxidation.

EXPERIMENTAL PART.

Analytical Methods.

THE ESTIMATION OF OXALIC ACID. There can be little doubt that much of the confusion which exists with regard to the origin and fate of oxalic acid in the organism is due to the employment of faulty methods of analysis. Of the various methods proposed for the estimation of oxalic acid, those associated with the names of Salkowski and Barth and Autenrieth are probably the most trustworthy, and the process employed in the present investigation is a modification of their methods. As large a quantity of urine as is available is heated for twenty minutes on the water-bath after the addition of about 5 per cent hydrochloric acid. The object of this procedure is to hydrolyze the oxaluric acid, for in the present state of our knowledge there appears to be no reason for differentiating between the free oxalic acid and that combined with urea. An excess of calcium chloride is next added and the liquid made fairly strongly alkaline with ammonia and allowed to stand in a warm place over night. The bulky precipitate is filtered off through a folded paper and well washed with boiling water. The filtrate should be preserved for a short time longer in order to give the finely divided calcium oxalate an opportunity to sediment. If necessary a second filtration is

made through Swedish filter paper and adhering precipitates are placed in a beaker and warmed with a *small* quantity of dilute hydrochloric acid and the liquid is then filtered through a small folded filter. The insoluble residue must be thoroughly washed by digesting with successive portions of hot water. The filtrate is concentrated on the water-bath to about 5 to 7 cc. and is then transferred to the extraction tube of an apparatus for continuous extraction with ether. The extraction with ether is carried out at a fairly rapid rate and usually was allowed to proceed for at least five or six hours. It is probable that two hours' extraction in an efficient apparatus is more than sufficient. About 20 cc. of water are added to the ethereal extract and the ether is then distilled off. The aqueous solution is filtered from any trace of flocculent insoluble residue and precipitated with calcium chloride and ammonia. The liquid is finally made decidedly acid with acetic acid and kept in a warm place for twenty-four hours. The precipitate of calcium oxalate is filtered off on a small Swedish filter paper, washed with hot water and either weighed as calcium carbonate or titrated with decinormal permanganate in the usual way. In a properly conducted experiment the two methods give identical results. If the calcium oxalate precipitated should be contaminated with any obvious amount of pigment, it is advisable to redissolve it in a few drops of hydrochloric acid and to reprecipitate with ammonia after adding a little calcium chloride. The addition of alcohol to the ether used for oxalic acid extraction as has been recommended, is disadvantageous and leads to the contamination of the calcium oxalate precipitate with impurities.

The rapidity and completeness of extraction of the oxalic acid by ether from aqueous solution was tested in the following experiment. Pure recrystallized ammonium oxalate equivalent to 17.8 mgms. of anhydrous oxalic acid was dissolved in 10 cc. of water to which a few drops of dilute sulphuric acid were added and the solution was then extracted with ether in a continuous extractor. The rate of extraction was such that the return flow of ether to the reservoir was just insufficient to form a continuous stream but gave a rapid succession of drops. After two hours and twenty minutes the receiver was changed and extraction allowed to proceed for a further period of three hours. The two

ethereal extracts and the aqueous residue were then separately analyzed for oxalic acid. The results were as follows:

Oxalic acid taken		17.8 mg.	
"	found in 1st ether extract (2½ hrs.)	17.8 mg.	Total 18.0 mg.
"	" 2d " (3 hrs.)	0.2	
"	" aqueous residue	00.0	

This result shows that under the prescribed conditions, oxalic acid in such quantities as would be found in urines, etc., may be extracted quantitatively in a few hours.

THE ESTIMATION OF ALLANTOIN. Methods for the estimation of allantoin have been described by Loewi[1] and by Poduschka[2] depending upon the precipitation of the allantoin in the form of silver and mercury compounds. The analytical results furnished by Loewi would lead us to conclude that the method was one of great accuracy. In four experiments in which allantoin was added to urine, the estimated amount differed from the actual, on an average, by less than two milligrams. In spite of the greatest care I have been completely unable to confirm Loewi's results and have come to the conclusion that the method is very misleading. It is unnecessary to go into the details of Loewi's method but it may be mentioned that it depends upon the removal of certain interfering substances by previous precipitation with mercurous nitrate, followed by the precipitation of the allantoin in the form of the silver salt by means of silver nitrate and magnesia. The magnesia is chosen in place of the more customary ammonia on account of the fact that an excess of the precipitant does not cause resolution of the precipitates. Loewi states with regard to the precipitate so obtained "Es hält von N-haltigen Körpern nur Allantoin and kann direct zur N-Bestimmung benutzt werden." In no single case have I been able to confirm this result. The silver precipitates have been carefully decomposed with sulphuretted hydrogen and freed from magnesia and it was found that in every case very considerable quantities of impurities were present. In every case substances precipitable by phosphotungstic acid in acid solution were present in large amount, and smaller quantities of bodies

[1] Loewi: *Loc. cit.*, p. 1.
[2] Poduschka: *Loc. cit.*

reacting in alkaline solution with diazobenzene sulphonic acid with production of a red coloring matter. The latter reaction is probably due to some substance containing the iminazol ring, such as histidin or the purin bases. Tyrosin was not present. Allantoin is not precipitated by phosphotungstic acid nor does it react with diazo salts with formation of colored products. In many cases the presence of urea was detected and it was found that this substance is precipitated by silver nitrate and magnesia to a considerable extent. The frequent freedom of the allantoin precipitate from urea depends upon the presence of ammonia in the urine. In many cases silver precipitates were obtained which contained large amounts of nitrogen but from which no trace of allantoin could be isolated in crystalline form either by Loewi's mercuric nitrate method or by other procedures. Many of the criticisms applied to Loewi's method hold equally for the other methods for the estimation of allantoin and it is, therefore, concluded that at present no satisfactory quantitative method is known. It therefore follows that results which are not based upon the actual isolation of the allantoin in crystalline form are of questionable value.[1]

The procedure adopted in the present experiments consisted in the precipitation of the allantoin along with urea and many other substances by means of mercuric nitrate. The acidity of the solution was kept low by addition of sodium carbonate. The mercury precipitate was filtered off, washed and decomposed with sulphuretted hydrogen. The filtrate was concentrated slightly and precipitated with silver nitrate and ammonia according to Poduschka's method. The silver precipitate was filtered off, washed, decomposed with sulphuretted hydrogen, and the filtrate concentrated to small bulk and examined microscopically for allantoin crystals. An estimation of the total nitrogen was also made by Kjeldahl's method but much reliance cannot be placed upon the figures so obtained as other substances are precipitated as well. If allantoin crystals can be detected in the

[1] A great many experiments were made to try and devise a satisfactory method for the estimation of allantoin but hitherto without success. The fact was established that allantoin could be quantitatively oxidized to oxalic acid by boiling alkaline permanganate and this fact may possibly be of analytical value.

syrupy solution, they may be freed from mother liquor by washing with a minimal amount of cold water and then recrystallized from boiling water. The method involves considerable loss of allantoin.

Ammonia determinations were made in the fresh urines according to Folin's method.

Experimental Results.

In almost every case, the results given are representative of a much larger number of experiments than those actually recorded.

1. THE EFFECT OF ACETATES UPON OXALIC ACID EXCRETION.

(*a*) Dog, 15 kilos, given 25 gm. of sodium acetate crystals dissolved in water, subcutaneously. The urine passed subsequently was clear and strongly alkaline. It effervesced vigorously on addition of acids.

Total oxalic acid in urine	before	injection	(24 hrs.)	0.0045	gram.
"	"	after	" (1st 24 hrs.)	0.0065	"
"	"	"	" (2d 24 hrs.)	0.0009	"

(*b*) Dog, 7 kilos, given solution of 25 gm. of sodium acetate crystals, subcutaneously.

Total oxalic acid in urine	before	injection	(24 hrs.)	0.0050	gram.
"	"	after	" (1st 24 hrs.)	0.0072	"
"	"	"	" (2d 24 hrs.)	0.0021	"

The slight increase in the oxalic acid excretion on the first day after the acetate injection is probably to be ascribed to the diuretic effects of the salts and is followed by a decreased excretion on the second day.

The experiments do not demonstrate the formation of oxalic acid as the result of acetate combustion in the normal organism.

2. EXPERIMENTS UPON THE FATE OF GLYCOLLIC ACID IN THE ORGANISM.

(*a*) Rabbit, 1550 grams, received subcutaneously 2.0 gm. of Kahlbaum's glycollic acid in the form of sodium salt. The rabbit had been fed for some days on bread and water. It received 100 cc. of water each day by mouth.

	Urine 48 hours before injection.	Urine 48 hours after injection.	Following 48 hours.
Volatile acids as formic acid	0.0165	0.0163	—
Oxalic acid	0.0009	0.0117	0.0049

(*b*) Same rabbit as Experiment (*a*). Two grams of glycollic acid as sodium salt given subcutaneously.

Oxalic acid, 48 hrs. before injection	0.0010
" " after "	0.0360

(*c*) Rabbit, 2 kilos, 2.0 grams of glycollic acid given subcutaneously as the sodium salt.

Oxalic acid 48 hrs. before injection	0.0056
" " after "	0.0400
" 24 hrs. later	0.0170
" " "	0.0067

(*d*) Dog, 10 kilos, 2 gm. of free acid in 100 cc. of water given by mouth in two equal portions about two hours apart. There was no vomiting.

	Total N.	N as NH_3.	N of NH_3 when total N=100.	Oxalic acid.
Urine 24 hrs. before acid given	5.40	0.191	3.54	0.0045
" " after "	4.07	0.192	4.72	0.0095
" " " "	4.12	0.165	4.00	0.0017

(*e*) Dog, 11 kilos, 1.11 gram free acid in 100 cc. of water given by mouth No vomiting.

	Total N.	N as NH_3.	N of NH_3 when total N=100.	Oxalic acid.
Urine 24 hrs. before acid given	3.17	0.121	3.32	0.0050
" " after "	4.93	0.223	4.52	0.0074
" " later	2.80	0.150	5.36	—
" " "	4.27	0.137	3.21	—

(*f*) Dog, 12 kilos, 3.34 grams of calcium glyoxylate given by mouth.

Oxalic acid in urine 24 hrs. before	0.0083
" " " after	0.0078
" " " later	0.0020

The results show that a very decided increase in oxalic acid excretion followed administration of glycollates to rabbits. Small doses of the free acid or calcium salt given by mouth to dogs does not produce marked oxaluria. No increase in formic acid (volatile acid) was observed. The ratio of nitrogen of ammonia to total nitrogen was increased by administration of free glycollic acid. It is probable that this is due to part of the glycollic acid escaping combustion and appearing unchanged in the urine. The fact that glycollic acid has been detected in urine after glycol administration is in accord with this view. No increase in allantoin excretion was observed.

3. EXPERIMENTS UPON THE FATE OF GLYOXYLIC ACID IN THE ORGANISM.

(*a*) One gram of the free acid was exactly neutralized with sodium carbonate and then diluted to 100 cc. and given by mouth to a rabbit weighing 1500 grams. The animal remained quiet for some time but no toxic symptoms were noted.

	Oxalic acid.	Volatile acid as formic acid.	Allantoin.
Urine 24 hrs. before	0.0030	0.0110	no crystals obtained.
" " after	0.0648	0.0648	" " "

(*b*) Same experiment except 1.5 grams of dry calcium glyoxylate given.

	Oxalic acid.	Volatile acid.	Allantoin.
Urine 48 hrs. before	0.0003	0.0136	minute trace.
" " after	0.0275	0.0152	no increase.

(*c*) Rabbit given 1.842 gram of dry calcium glyoxylate subcutaneously.

Oxalic acid in urine 48 hrs. before	0.0050
" " 72 hrs. after	0.0778

(*d*) Rabbit given 1.25 grams of methylglyoxylate in 100 cc. of water by mouth.

	Oxalic acid.	Allantoin.
Urine 36 hrs. before	0.0058	doubtful trace.
" 48 " after	0.0297	" "

(*e*) Dog, 17 kilos, given 2.8 grams of free glyoxylic acid in 130 cc. of water by mouth. After a short time vomiting occurred and 0.5 gram of acid was ejected. No further vomiting took place.

	Oxalic acid.	Allantoin.	Total N per 100 cc.	N as NH_3 per 100 cc.	N as NH_3 when total N = 100.
Urine 24 hrs. before	0.0121	trace	3.25	0.138	4.24
" " after	0.0170	no crystals	1.29	0.046	3.56
" " later	0.0045	no crystals	—	—	—

(*f*) Dog, 9 kilos, given 2.0 grams of free glyoxylic acid in 150 cc. water in two portions five hours apart. No vomiting occurred.

	Total N per 100 cc.	N as NH_3 per 100 cc.	N as HN_3 when total N = 100.
Urine 24 hrs. before (faint acid)	5.98	0.274	4.58
" " after (neutral)	4.54	0.189	4.16
" " later (faint acid)	5.40	0.191	3.54

The results show that a marked oxaluria follows the administration of salts of glyoxylic acid in the case of rabbits. With

dogs the oxalic acid excretion may be scarcely increased. No evidence was obtained of the formation either of allantoin or of formic acid. The administration of the free acid was not followed by an increase in the $NH_3 : N$ ratio. It is improbable that any material amount of glyoxylic acid is excreted unchanged.

4. EXPERIMENTS UPON THE FATE OF OXALIC ACID IN THE ORGANISM.

(*a*) Rabbit, 1600 grams; 3.3 cc. of ammonium oxalate solution (equivalent to 0.0990 gram oxalic acid) injected subcutaneously. The rabbit was fed on bread and water.

Oxalic acid in urine, 24 hrs. before	0.0030
" " " after	0.0135
" " " later	0.0051

(*b*) Rabbit, 1550 grams, ammonium oxalate equal to 0.051 gram of oxalic acid injected subcutaneously.

Oxalic acid in urine 24 hrs. before	0.0015
" " " after	0.0077
" " " later	0.0030

(*c*) Rabbit, 1000 grams, ammonium oxalate solution equivalent to 0.297 gram oxalic acid injected subcutaneously. Rabbit died after 48 hours.

Oxalic acid in urine 24 hrs. before	0.0078
" " " after	0.0312
" " " later	0.0135

The results show that moderate amounts of oxalic acid are almost completely burnt in the organism—larger amounts are not so completely oxidized. Similar results were obtained with dogs.

5. OXALURIA PRODUCED BY GLYCOL ADMINISTRATION.

(*a*) Rabbit, 2250 grams, given 2.5 grams of glycol diluted with water, injected subcutaneously.

Urine 48 hrs. before	0.0040	oxalic acid.
" 72 " after	0.0600	" "

(*b*) Repetition of the preceding experiment; 2.5 grams of glycol by subcutaneous injection.

Oxalic acid in urine 72 hrs. after 0.0750

The results show a marked oxaluria as the result of glycol administration, confirming the previous results of Paul Mayer and Pohl.

6. EFFECT OF GLYCOL ADMINISTRATION UPON OXALIC ACID EXCRETION.

(*a*) Rabbit, 1550 grams; previous diet of bread and water; a solution of 2.5 grams of glycocoll injected subcutaneously. Rabbit starved 48 hours after injection; 100 cc. of water per day given by mouth.

Urine 48 hrs.	before	0.0049 gram oxalic acid.
" "	after	0.0045 " "

(*b*) Repetition of same experiment.

Urine 48 hrs.	before	0.0015 gram oxalic acid.
" "	after	0.0035 " "

The results show that in the case of rabbits no appreciable oxaluria follows the administration of glycocoll by subcutaneous injection.

7. EFFECT OF GELATIN-FEEDING UPON OXALIC ACID EXCRETION.

(*a*) Fifty grams of gelatin were dissolved in 1400 cc. of water with the addition of a little sherry and sugar. It was consumed by a healthy man within two hours. Before and after the experiment care was taken to avoid food which would be liable to yield oxalic acid.

Urine 24 hrs.	before	0.0008 gram oxalic acid.
" "	after	0.0190 " "

(*b*) Repetition of same experiment; 40 grams of gelatin consumed.

Urine 24 hrs.	before	0.0270 gram oxalic acid.
" "	after	0.0332 " "

(*c*) Repetition of same experiment; 40 grams of gelatin consumed.

Urine 24 hrs.	before	0.0093 gram oxalic acid.
" "	after	0.0160 " "

The results are similar to those obtained by other observers and appear to show that a slight oxaluria does follow the consumption of large quantities of gelatin, at least in the case of man.

ON THE REDUCTION OF BARIUM SULPHATE IN ORDINARY GRAVIMETRIC DETERMINATIONS.

By OTTO FOLIN.

(Received for publication, February 4, 1907.)

By copying the title of my recent paper "On Sulphate and Sulphur Determinations"[1] (a device rarely adopted except in purely controversial and polemical communications) S. F. Acree[2] has rather vigorously called attention to his criticisms of my brief statement that barium sulphate precipitates are not ordinarily reduced when ignited together with the filter paper upon which they have been collected.

Acree maintains that barium sulphate is nearly always reduced when filter paper is employed in sulphate determinations, and that the reduction is often extraordinarily large. My statement was made on the basis of what appeared to me an adequate number of tests, but without particular emphasis, because I still found it necessary for good results to use Gooch crucibles instead of filter paper. Since the appearance of Acree's paper the point has again been tested, and as before only a variable mechanical loss and no appreciable reduction of barium sulphate has been found.

Forty cc. of approximately tenth normal sulphuric acid gave 470.6 mg. barium sulphate as the average of three closely agreeing determinations by the help of Gooch crucibles.

1. The precipitate from 40 cc. of the above solution collected on "black ribbon," S. and S. filter (No. 589, diam. 9 cm.), dried, and ignited in a platinum crucible, weighed 465.5 mg. After adding and carefully boiling off 3 cc. of conc. sulphuric acid the residue weighed 465.7 mg.

2. Another precipitate from 40 cc. of the solution was collected on three filters (folded together). After carefully drying, charring and burning the weight of the precipitate was found to be 465.5 mg. After treatment with six drops of 50 per cent sulphuric acid the weight remained unchanged (465.6 mg.), nor did a second treatment with 1 cc. of conc. sulphuric acid yield a different result (weight 465.3 mg.)

[1] This *Journal*, i, p. 131.

[2] This *Journal*, ii, p. 135.

3. A third precipitate collected on two filters weighed 469.8 mg. After sprinkling 1.005 gm. of pure grape sugar over this precipitate and carefully charring and burning the mixture a loss of 1.3 mg. due to reduction was obtained (weight 468.5 mg.) The residue was then moistened with water and three drops sulphuric acid and re-heated. The loss due to reduction was recovered (weight 470.2 mg.), and the escape of some hydrogen sulphide was noted.

The experiments 1 and 2 recorded above were the only ones which gave precipitates the original weights of which did not *exclude* any appreciable reduction of sulphate by the burning of the filters. Several other determinations were made by the use of filter paper, but in these the amount of barium sulphate found corresponded so nearly to the theoretical figure (470.6) as to be well within the limits of unavoidable experimental error.

The final three determinations made by the use of filter paper, for example, gave the following values: (1) 469.6; (2) 470.7, (3) 470.2.

From these experiments it would appear that barium sulphate is even more resistant to the action of reducing agents than I had previously supposed and consequently I can not concede the validity of Acree's criticism of my earlier statement. The results of Acree and his students furnish, of course, the proof, if any proof is necessary, that it is possible to reduce barium sulphate to sulphide at ignition temperatures; but, in the light of positive experience to the contrary, they do not prove that it is difficult to avoid such reduction.

I have also been unable to confirm Acree's statement that it is difficult to convert barium sulphide into the sulphate by treatment with sulphuric acid. His attempt at explaining this alleged difficulty on the basis of "occlusion" is scarcely tenable in view of the fact that barium sulphate is very soluble in hot concentrated sulphuric acid. In his experiment No. 3, for example (p. 138), he repeatedly added enough sulphuric acid (3 cc.) to dissolve the entire barium sulphate precipitate (.45 g.)

ON THE OCCURRENCE AND FORMATION OF ALKYL UREAS AND ALKYL AMINES.

By OTTO FOLIN.

(*From the Chemical Laboratory of McLean Hospital, Waverley, Mass.*)

(Received for publication, February 4, 1907.)

In connection with feeding experiments with creatin made during the past year it seemed desirable to determine whether any of the missing creatin could have appeared in the urine in the form of methyl urea or methyl amine. The results were negative, but in the course of the investigation I found that all human urines contain certain small amounts of a substance which might very well be methyl urea, and that some urines, particularly typhoid urines, also contain appreciable quantities of methyl amine.

For the detection of these substituted ammonia derivatives I have used Hoffmann's iso-nitril reaction. The test is made as follows: The ammonia-containing distillates obtained from urea and from ammonia determinations are slightly acidified and concentrated by boiling to 50 or 75 cc. The solutions are then transferred to small Kjeldahl flasks, and further concentrated to a volume of from 5 to 15 cc. To the remainder are added about 25 cc. of saturated alcoholic potash solution and a few drops of chloroform. On gently boiling these mixtures the intense, disagreeable odor of iso-nitril is obtained. The odor becomes particularly characteristic after most of the ammonia has been driven off and the remaining solution has cooled.

The possible occurrence of methyl urea in urine was the subject of considerable investigation and discussion some twenty-five years ago, but the positive results recorded by some, notably by Shiffer, were never accepted, and in recent years this problem has, as far as I know, received no further attention.

Shiffer's results may indeed appear conclusive (*Zeitschr. f. physiol. Chem.*, iv, p. 237, 1880) to the casual reader, for he claimed to have shown that the methyl amine group of creatinin did not interfere with

Hoffmann's reaction as he applied it. This contention of Shiffer's was, however, evidently never accepted; and rightly so, for creatin and creatinin do split off methyl amine when boiled in caustic soda or potash solutions.

The question of the presence or absence of traces of methyl urea in normal urine is perhaps not a very important one, except in so far as it is a part of the broader question, as to the origin of other methyl amine groups in urine, such as those of methyl purins and of creatinin. If all methyl amine groups in urine are derived from preformed methyl amine groups in the food, as Koch,[1] for example, has recently attempted to explain the formation of creatinin from the trimethyl amine of lecithin, then one can attach but little importance to the methyl urea and methyl amine of urine, for then they may represent only a certain excess of such methyl amine groups in the food just as ordinary urea to a large extent represents the unnecessary nitrogen of the amido acids. On the other hand, if the methyl amine groups in urine are not necessarily derived from preformed methyl amine groups in the food but can just as well be derived from the abundant amido acids of ordinary proteins, then the occurrence of methyl urea and of methyl amine salts is important because then we have no reason to assume that they may not occasionally occur in very large quantities.

The main point which I wish to bring out in the present communication is that methyl or other alkyl amine groups are obtainable from ordinary amido acids, and therefore from all proteins.

The formation of ammonia and through ammonia of urea, in protein catabolism is in all probability more a matter of hydrolysis than of oxidation.[2] Similarly in the decomposition of protein derivatives by boiling sulphuric acid, as in the Kjeldahl method for the determination of nitrogen, the formation of ammonia is undoubtedly the result of hydrolysis rather than of oxidations.[3]

It occurred to me that there is no binding reason why in hydrolytic cleavages of this kind the nitrogen atoms should be quantitatively separated from the carbon atoms to which they are

[1] W. Koch: *Amer. Journ. of Physiol.*, xv, p. 15, 1905.
[2] Folin: *Amer. Journ. of Physiol.*, xiii, p. 124, 1905.
[3] Folin: *Zeitschr. f. physiol. Chem.*, xli, p. 239, 1904.

attached. Schematically such cleavage can just as well be made to yield methyl amine as ammonia.[1]

$$(1)\quad \equiv C{-}CH_2\boxed{NH_2+H}OH$$

$$(2)\quad \equiv C{-}\boxed{CH_2NH_2+H}OH$$

The experiments undertaken to test the correctness of this point of view gave at once positive results. The distillates obtained from ordinary Kjeldahl determinations of the total nitrogen, when concentrated and further treated as described above, give invariably intense iso-nitril reactions. Witte's peptone, urine, creatin, creatinin, glycocoll, aspartic acid, and hippuric acid, all give it,[2] and it makes no appreciable difference whether the decomposition is produced by sulphuric acid alone or by sulphuric acid plus copper sulphate or mercury.

These results prove that methyl amine (or other alkyl amines) can be formed by hydrolysis from ordinary amido acids, and in the light of this fact there is, I think, no reason for assuming any genetic relationship between the methyl amine groups of the food and those of the urine where experimental evidence in favor of such a relationship is wanting.[3]

Further, if methyl amine, and therefore also methyl urea, *can be formed* by the catabolism of ordinary amido acids, then it is neither impossible nor particularly improbable that a systematic investigation of the urines from a large number of different kinds of animals would show that some contain relatively large quantities of such substituted ammonia derivatives; nor is their exclusive formation and elimination as abnormal metabolic products in diseases excluded.

[1] The fact that the Kjeldahl process ordinarily gives perfectly accurate analytical results does not prove that the nitrogen has been quantitatively split off as ammonia, because if any methyl amine were formed and not subsequently oxidized it would come over together with the ammonia in the distillation and would act as ammonia in the titration.

[2] Pure urea, as was to be expected, gives only ordinary ammonia.

[3] The recently discovered fact that the human organism does not even convert food creatin directly into urinary creatinin (see *Festschrift für Olof Hammarsten*, iii, 1906) illustrates how fallacious such assumptions may be.

The occurrence of tetra- and pentamethylene diamines in some cases of cystinuria may be considered as an illustration of such an abnormal metabolism. Lysin and arginin are in all probability, as C. Neuberg[1] has pointed out, the direct precursors of these diamines, though no one has yet been able to verify Neuberg's remarkable, not to say peculiar, experimental results.

Unfortunately I have not yet been able to find a quantitative method for the determination of methyl amine in the presence of ammonium salts; and without such a method it is not possible to determine with any degree of certainty just what rôle the formation of methyl amine and methyl urea may play in protein metabolism. The data which I have so far obtained in attempts at quantitative estimations indicate that certainly not more than 2 or 3 per cent of the urea-nitrogen (corresponding to 4 to 6 per cent of the urea) is normally eliminated on nitrogen-rich diets as methyl urea. On low nitrogen diets the methyl urea elimination diminishes but never wholly disappears. On low nitrogen diets in typhoid fever the ammonia elimination is very high, and this ammonia contains, I think, an admixture of at least 5 or 6 per cent of methyl amine.

[1] C. Neuberg: *Zeitschr. f. physiol. Chem.*, xliii, p. 352, 1904.

ON THE SYNTHESIS OF PROTEIN THROUGH THE ACTION OF TRYPSIN.

By ALONZO ENGLEBERT TAYLOR.

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(Received for publication, March 12, 1907.)

The attempts to verify in biological material the laws of mass action, equilibrium and partition have not been uniformly successful. In some instances the verifications were easily accomplished, in others success could not be attained. The difficulties are resident in the complexities of the systems under experimentation and in the lability of the reactions concerned. The same comments apply to the verifications in the fermentations of the laws of catalysis. In one particular, however, in the exceedingly important matter of synthesis by ferment action, the experimental success has been unexpectedly uniform. Following the first report of a successful reversion by Hill,[1] successful experiments were reported by Kastle and Loevenhart,[2] Bernizone,[3] and Emmerling,[4] and these have been followed since by those of Fischer and Armstrong,[5] Pottevin,[6] Wroblewski,[7] Acree and Hinkins,[8] and Taylor.[9] For the fats the relations seem clear. Esters of both glycerine and the monatomic alcohols and the

[1] Hill: *Journ. of the Chem. Soc.*, lxxiii, p. 634; *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 1380; Emmerling: *Ibid.*, xxxiv, pp. 600, 2206.

[2] Kastle and Loevenhart: *Amer. Chem. Journ.*, xxiv, p. 491.

[3] Bernizone: *Atti de Soc. lig. di Scien. nat. e Geograf. Genoa*, xi, p. 327.

[4] Emmerling: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 3810.

[5] Fischer and Armstrong: *Ibid.*, xxxv, p. 3144.

[6] Pottevin: *Compt. rend. de l'Acad. d. sci.*, cxxxvi, p. 1152; cxxxviii, p. 378.

[7] Wroblewski: *Journ. f. prakt. Chem.*, N. F., lxiv, p. 41.

[8] Acree and Hinkins: *Amer. Chem. Journ.*, xxviii, p. 370.

[9] Taylor: *Univ. of Cal. Publications*, Pathology, i, p. 33.

acids of the series $C_nH_{2n}O_2$ (and of oleic acid also) may be formed under appropriate conditions through the action of lipase. With the carbohydrates the relations are less simple. All the successful reversions definitely known comprise the formation of disaccharides or glucosides. Cremer[1] has described the formation of glycogen from d-glucose, and Roux, Maquenne, Fernbach and Wolff[2] have adduced data tending to show that starch may be formed from glucose through the action of amylase; in both instances, however, the proper chemical confirmatory evidence has not been supplied, though the general evidence tends to support the inferences of reversion. For the proteins no reversions have been reported. Three years ago I[3] published the results of a series of negative attempts to form protamin from amido-acids under the action of trypsin. Later I attempted to form the synthetic peptides of Fischer from the appropriate amido-acids under the influence of trypsin, again to no result. Recently Abderhalden and Rona[4] have published the details of a series of similarly negative results. I am now able to report a successful synthesis of protamin from amido-acids through the action of trypsin.

I have again employed protamin, despite the previous negative results, because it was clear that if the proper conditions could be secured, the use of such a simple protein presented a better prospect of a positive result than could be expected with the use of a higher and more complexly formed protein. An investigation of the relations of equilibrium in the hydrolysis of protamin suggested that the maximum of concentration of the amido-acids should be employed. I had in most of the earlier experiments used the amido-acids in the form of the sulphates; that is, the protamin sulphate was completely digested, and then the attempt was made to regenerate protamin sulphate. It was clear from the theory of the reaction that the strong combination of the amido-acids with the sulphuric acid was unfavorable

[1] Cremer: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 2062.

[2] Roux, Maquenne, Fernbach and Wolff: *Compt. rend. de l'Acad. d. sci.*, cxxxvii, p. 718; cxxxviii, pp. 819, 849; cxxxix, p. 1217; cxl, pp. 95, 1303, 1403, 1547, 1067.

[3] Taylor: *Univ. of Cal. Publications*, Pathology, i, p. 65.

[4] Aberhalden und Rona: *Zeitschr. f. physiol. Chem.*, xlix, p. 31.

to the reaction of synthesis. In the present experiment, therefore, the amido-acids were used in the free state or as carbonates. When a solution of protamin sulphate is digested with trypsin the cleavage is usually complete. If the mass of ferment be small, a trace of the substrate will remain. It is possible however, to secure the apparent station of equilibrium that is to be observed in fermentations in general, in the following manner. A saturated solution of protamin sulphate is digested with trypsin, and from time to time fresh portions of the substrate in powder form are added. A time will come when an undigested fraction of the substrate remains in the system, and cannot be digested, even by the addition of fresh portions of ferment. If now the system be diluted, the digestion will recommence. The station of equilibrium is obviously determined by the concentration of the products of the reaction. The products of the hydrolysis of protamin, the amido-acids, are very soluble in water; the substrate is not very soluble in water. Under these circumstances, it is clear that unless some relation intervenes to shift the station of equilibrium or the protamin is removed from the system, the synthesis of a large amount of the protamin cannot be anticipated, even though the concentration of the amido-acids be high.

Under our present conditions of experimentation at least, all ferment reversions are reactions of slow velocity. Trypsins of higher origin are, however, labile substances, and in solution rapidly undergo hydrolysis which destroys the enzymic property. Under these circumstances I cast about for a more stable trypsin. From the liver of the star-fish I obtained a trypsin that was very active, but not particularly resistant to hydrolysis. From the liver of a large Pacific coast clam (*Schizothærus Nuttallii*) I have obtained a trypsin that is very resistant to hydrolysis. This ferment, in the form of a glycerine extract of the tissue, I have used in the experiment to be described.

The protamin sulphate used in these experiments was derived from the *Roccus lineatus*. It has, as I have previously determined, the composition $C_{30}H_{60}N_{17}O_6 \cdot 2\ H_2SO_4$. Four hundred grams of the sulphate were dissolved in about 15 liters of warm water, the solution made alkaline, and digested with trypsin until the solution was miscible with five volumes of acidulated alcohol,

and bore saturation with sodium chloride without the production of any opacity or precipitation.[1] It was clear from these tests that the digestion was completed, and that the fluid then represented the solution of the amido-acids that are the products of the hydrolysis of protamin. The solution was then heated to the boiling point, the sulphuric acid precipitated by the addition of barium chloride, the slight excess of barium precipitated by carbon dioxide, filtered hot, and the filtrations repeated until the filtrate was clear. The filtrate remained clear after cooling, and gave an alkaline reaction. The fluid was then concentrated on the water-bath until a sample on cooling showed beginning precipitation. This cooled fluid then represented a concentrated solution of free amido-acids and their carbonates, the products of the cleavage of protamin. Some 700 cc. were preserved as a control, and 4 liters were employed for the reversion experiment. To this were added 300 cc. of the glycerine extract of the clam livers; this mixture could be added to 4 parts of acidulated alcohol without the production of any opacity. Twenty grams of toluol were then added, the bottle sealed and set aside at room temperature. As time passed the contents of the bottle became opalescent, then cloudy and finally a small white precipitate settled upon the bottom of the bottle, the supernatant fluid remaining cloudy. Five months after the experiment was begun, the container was opened, and the contents tested bacteriologically, with negative results. To the contents of the bottle sulphuric acid was then added until the reaction was acid, whereby the precipitate and cloudiness disappeared. The solution was then heated to the boiling point, filtered, and the filtrate poured into four volumes of acidulated absolute alcohol, with the production of a white precipitate. This precipitate was collected upon a filter paper, washed with alcohol, dissolved in water, filtered, again precipitated with alcohol and this procedure repeated four times. The final precipitate was dried with alcohol and ether, and dehydrated in the desiccator at 90° C.

[1] The test with alcohol was made as follows: 200 cc. of the solution were acidulated with sulphuric acid and then mixed with one liter of absolute alcohol. The solution remained clear. After several days, the solution still remaining clear, the alcohol was removed by heating, and the remaining solution returned to the original liquor.

It weighed 1.8 gram. Possibly one-fourth as much had been lost in the operation of isolation and purification. This powder is soluble in about thirty-five parts of water, is precipitated by three parts of alcohol, and is salted out of solution by a sodium chloride concentration of 10 per cent. It is digestible by trypsin, resistant to pepsin. The elementary analysis gave the following figures:

0.2752 gm. of substance yielded	0.3802 gm. CO_2 and 0.1708 gm. H_2O.	
0.2274 " " " "	0.0556 " nitrogen.	
0.2378 " " " "	0.0593 " "	
0.087 " " " "	0.0219 " "	
0.1592 " " " "	0.0399 " "	
0.4358 " " " "	0.2146 " $BaSO_4$.	

The theoretical values and the analytical results are as follows:

Calculated for $C_{30}H_{60}N_{17}O_6.2H_2SO_4$:	Found:
C = 37.85 per cent	37.68 per cent
H = 6.72 " "	6.89 " "
N = 25.13 " "	24.45, 24.93, 25.17, 25.06 } Average 24.9 per cent
H_2SO_4 = 20.60 " "	20.69 per cent.

The values for the analyses are in good agreement with those demanded by the formula for protamin sulphate and there can be no doubt that the substance is protamin sulphate.

The portion of the solution of amido-acids that had been preserved for a control was found to be sterile and had undergone no change.[1] The glycerine extract, a portion of which was used in the experiment, was found to be active after the completion of the experiment.

The successful result of the experiment when contrasted with the negative results in the previous experiments must be due to one of two factors, or to both; to the use of the concentrated solution of the free amido-acids and their carbonates; and to the use of a stable trypsin. The velocity of the synthesis is very slow. From about 400 grams of the amido-acids some 2 grams of protamin were synthesised in five months. All the reported

[1] The test was done as before described: 200 cc. of the solution were acidulated with sulphuric acid and mixed with one liter of absolute alcohol; no opacity or precipitation was produced.

syntheses have been slow. When the reactions of the reversions are better understood, this will be an important question to be investigated according to the mathematical principles of physical chemistry; it has, however, no bearing upon the theory or fact of the reversion.

The meaning of the experiment is clear. Like all proteins, protamin is composed of amido-acids. The digestion of protamin is an hydrolysis; the synthesis of protamin from the amido-acids is a condensation. The reaction runs:

$$\text{Protein} + \text{Water} \leftrightarrows \text{Amido-acids.}$$

Under appropriate conditions trypsin, in accordance with the theory, will accelerate the reaction in either direction, in the direction of synthesis as well as in the direction of hydrolysis. From the point of view of general theory, the formation of the blood protein from the products of the digestion of protein is to be interpreted as a reversion. This experiment furnishes a confirmation of this theory.

The general medical and biological public is not as yet fully conversant with the theory of the reversed reaction, or with the theory of the laws of mass action and equilibrium, as has been well illustrated by the reception that has been accorded in the medical world to the quantitative measurements and calculations of Arrhenius in the question of the toxins and antitoxins, as well as by the attitude to the theory of catalysis displayed by all the text books on fermentation. It is the conviction of the writer that this, for the matter of the reversed reactions here under consideration, is due to the fact that the energy relations concerned in such a system are not understood. The reaction is progressing to a station of equilibrium, and there is always a certain zone of reaction to either side of the station of equilibrium. For some systems, as in the reaction

$$\text{Ester} + \text{Water} \leftrightarrows \text{Alcohol} + \text{Fatty acid,}$$

the reaction on either side of the station of equilibrium is easily demonstrable; in many others, however, the station of equilibrium is so near to a completed reaction that special conditions of experimentation are required to demonstrate that the reaction is limited and reversible. In every such reaction there is a driv-

ing force and an internal chemical resistance. When now a catalysor or a ferment accelerates such a reaction, it does so solely through a reduction in the internal chemical resistance; the driving force remains the same. Obviously, therefore, a catalysor or ferment can accelerate a reversion or synthesis to the station of equilibrium even though that reaction be an endothermic one. In whatever direction the reaction is progressing under the conditions of the experiment, whether it be building up or breaking down, the ferment must accelerate the reaction. The term synthesis through ferment action is, in the direct sense, a misnomer. The ferment of course simply accelerates the reaction of synthesis. The synthesis is a reaction *per se;* the ferment simply so accelerates the velocity as to make the result apparent within experimental time.

(*Postscript made at the time of proofreading, April* 24, 1907.)

When in January I realized that a positive result had probably been attained, another control experiment was arranged to exclude the possible objection that some other substance than trypsin in the glycerin extract might have been the cause of the reversion. From the theoretical point of view such an objection was most improbable; nevertheless, in view of the criticisms to which reversion experiments are subjected, the direct experiment was carried out. Four hundred cubic centimeters of the original solution of concentrated products of the protamin digestion were mixed with some 40 cc. of the glycerin extract that had been previously heated to the boiling point for ten minutes to destroy the activity of the ferment. Toluol was then added and the mixture set aside. After more than three months this test was analyzed. The mixture was sterile. Two hundred cubic centimeters of the mixture were acidulated with sulphuric acid and mixed with one liter of absolute alcohol; the liquid remained clear, no opacity whatever resulted. This experiment was identical with the positive experiment except in that the ferment had been inactivated by heating. The experiment and the several controls may be tabulated as follows:

Original digestion-solution + alcohol = no opacity or precipitation.

Concentrated digestion-solution + ferment extract + alcohol = no opacity or precipitation.

Concentrated digestion-solution after 5, respt. 8 months + alcohol = no opacity or precipitation.

Concentrated digestion-solution + ferment extract, after five months, + alcohol = precipitate, protamin.

Concentrated digestion-solution + boiled ferment extract, after three months, + alcohol = no opacity or precipitation.

These controls indicate that the reversion must have been associated with some thermolabile substance in the ferment extract. There is every reason to attribute this result to the trypsin which the extract was known to contain. The control experiments indicate also that the protamin recovered must have originated in the particular experiment, and cannot have been an undigested residue, since under such circumstances it would have been recoverable from the controls, whereas they were all negative to the method with which protamin was isolated from the test with the active ferment.

NOTE ON THE SYNTHESIS OF A PROTEIN THROUGH THE ACTION OF PEPSIN.

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(Received for publication, April 8, 1907.)

In a recent paper Taylor[1] has described the synthesis of a protein (protamin) through the action of a trypsin obtained from the liver of the soft-shelled California clam. The concentrated products of the tryptic digestion of 400 grams of protamin sulphate were converted into the carbonates and subjected to the action of the trypsin; at the end of five months about 2 grams of protamin (weighed as sulphate) were obtained from the solution. By pursuing a method in some respects similar to the above I have succeded in synthesising one of the first products of the peptic digestion of casein, a substance, or mixture of substances, to which the name "Paranuclein" has been applied.[2]

Four hundred cc. of $\frac{N}{50}$ potassium hydroxide were "saturated" with casein[3] and the solution subjected to the action of pepsin for several days at 40° C. It was then heated to 100° C. for about 10 or 15 minutes to destoy the ferment and filtered while hot. The solution was then evaporated on a water-bath to a volume of about 70 cc. This concentrated solution of the products of the peptic digestion of casein is a clear brown syrup which is strongly acid and gives no precipitate or opalescence upon the addition of acetic acid or upon the addition of acetic acid in excess after having previously rendered the solution alkaline by the addition of potassium hydroxide. Both casein and paranuclein are therefore absent from the solution. To 70 cc. of this solution were added

[1] *Univ. of Cal. Publications*, pathology, i, p. 343, 1907. This *Journal*, iii, p. 87, 1907.

[2] For literature, consult Gustav Mann, *Chemistry of the Proteids*, 1906, pp. 395-396.

[3] T. Brailsford Robertson: This *Journal*, ii, p. 337, 1907.

30 cc. of a concentrated (approximately 10 per cent) solution of Grübler's pepsin which had previously been filtered through rapid filtering paper. The mixture of the two solutions was a clear brown syrupy fluid which gave no trace of a precipitate with acetic acid either before or after neutralization with alkali. The mixture was set aside at 40° C. in the presence of excess of toluene to prevent bacterial infection. Within two hours a thick white precipitate had formed in the solution; after 48 hours the solution was filtered and the precipitate thus collected on the filter was dissolved in a minimal amount of sodium hydroxide and the filter so arranged that the alkaline solution dropped directly into water acidified with acetic acid. The precipitate thus obtained was reprecipitated twice and was washed by decantation several times. Finally it was collected on a filter, washed with alcohol and with ether in a desiccator and dried at 60° C. In this way 1.02 grams of a friable, grayish-white, hygroscopic powder were obtained; I do not think that more than 10 per cent was lost in the process of purification. The original filtrate was tested for casein by rendering alkaline with sodium hydroxide and then adding excess of acetic acid—a very slight precipitate was formed, too small in amount to collect and purify.

Paranuclein was prepared by partly digesting sodium caseinate with pepsin, filtering off the precipitate, dissolving in sodium hydroxide and precipitating with acetic acid. The paranuclein was reprecipitated twice, washed with alcohol and ether in a desiccator and dried at 60°. A powder exactly similar in appearance to the synthesised substance was thus obtained. The physical properties of the precipitates with acid, bulky, flocculent and settling rapidly, were identical in both substances.

The synthesised substance and the paranuclein were both analyzed for phosphorus by Neumann's method[1] with the following results:

0.1014 gm. of substance yielded 0.001665 gm. P_2O_5
0.1010 " " " 0.001598 " P_2O_5

Hence P_2O_5 = (1) 1.64 per cent. (2) 1.58 " " } Average—1.61 per cent.

0.1027 gm. of paranuclein yielded 0.004312 gm. P_2O_5
0.0988 " " " 0.004099 " P_2O_5

[1] Neumann: *Arch. f. Anat. und Physiol.*, p. 159, 1900.

Hence P_2O_5 = (1) 4.2 per cent. (2) 4.15 " " } Average—4.175 per cent.

Previous observers agree in stating that the percentage composition of paranuclein varies very greatly with the circumstances under which it is prepared,[1] the percentages of phosphorus which have been found in various preparations varying from 0.88 to 6.86.[2] This fact leads us to suspect that the substance which has been termed paranuclein is, in reality, a mixture of at least two substances and the hypothesis which has suggested itself to me is that during the hydrolysis of casein by pepsin an insoluble substance of high phosphorus content is first formed, that this substance splits off a soluble phosphorus-containing moiety leaving another substance, insoluble in acid, of lower phosphorus content, and that this second substance is in its turn attacked and splits up into soluble substances. That this explanation is probably the correct one, although, of course, several such steps may be involved, is shown by the following experiment.

One gram of the paranuclein containing 4.175 per cent of P_2O_5 was dissolved in 400 cc. of 0.045 N $Ca(OH)_2$ and stood at 40° C. for 12 hours; acetic acid was then added in excess and the precipitate washed and dried in the manner described above. This substance, which we may designate, Paranuclein A, on analysis for P_2O_5, gave results as follows:

0.1071 gm. yielded 0.00174 gm. P_2O_5
0.0957 " " 0.00131 " P_2O_5

Hence P_2O_5 = (1) 1.62 per cent. (2) 1.4 " " } Average—1.51 per cent.

Only a little over 0.2 gm. of this substance was obtained from the gram of paranuclein originally dissolved in the lime-water.

This splitting off of phosphorus from paranuclein, particularly in alkaline solution, has been commented on by other observers.[3]

The substance which is obtained synthetically, as described above, resembles very closely Paranuclein A, both in physical properties and in percentage of P_2O_5. The synthesised substance is practically insoluble in acids, is readily soluble in dilute alkali, precipitates protamin from a 1 per cent solution of the sulphate

[1] See literature quoted in Gustav Mann, *loc cit.*

[2] W. v. Moraczewski: *Zeitschr. f. physiol. Chem.*, xx, p. 28, 1895.

[3] Salkowski und Hahn: *Arch. f. d. ges. Physiol.*, lix, p. 225, 1895.

at a reaction just alkaline to phenolphthalein (at which reaction both the protamin and the paranuclein remain in stable solution when not mixed), and a 2 per cent solution in $\frac{N}{100}$ sodium hydroxide is precipitated by $\frac{M}{10}$ ferric ammonium sulphate; these being all well known properties of paranuclein.[1]

The synthesised substance also resembled my preparation of paranuclein in the following properties: in approximately 2 per cent solution in $\frac{N}{100}$ sodium hydroxide it gives the xanthoproteic, Millon's, Adamkiewicz and the biuret (violet) reactions; it is precipitated by cupric chloride (1 vol. of $\frac{N}{1}$ to 100) and by zinc chloride but not by mercuric chloride (5 vols. of $\frac{N}{10}$); it is precipitated by picric and by tannic acids, but the precipitate redissolves on rendering the solution alkaline; it is not precipitated by the addition of five volumes of absolute alcohol; and the precipitate at first produced by the addition of acetic acid is soluble in considerable excess of glacial acetic acid.

If no pepsin be added to the concentrated solution of the peptic digestion of casein, prepared as described above, the solution, after keeping for three weeks at 40° C. remains perfectly clear and homogeneous and gives no tests for paranuclein or for casein. A 10 per cent, filtered solution of Grübler's pepsin, on standing for the same period at 40° also remains perfectly clear and homogeneous. Yet these two solutions when mixed in the proportion of 1 vol. of ferment to 5 vols. of the solution of the casein products, gave a voluminous precipitate which remained permanent throughout the period of three weeks.

The experiment has been repeated a number of times, always resulting in the production in the solution of a precipitate resembling in properties that which is described above. All the experiments were carried out in the presence of an excess of toluene (several drops to 50 cc.).

Further experiments are in progress.

CONCLUSIONS.

1. The substance, derived from casein by incomplete digestion with pepsin, which has been termed paranuclein is probably

[1] Salkowski and Hahn: *loc. cit.* Milroy: *Zeitschr. f. physiol. Chem.*, xxii, p. 307, 1896.

a mixture of at least two substances, the one containing a high percentage of phosphorus and the other, in other respects similar in properties to the former substance, containing a much smaller percentage of phosphorus. Paranuclein containing 4.175 per cent of P_2O_5 digested with lime-water for twelve hours at 40° C. yields a small quantity of a substance similar in properties to the paranuclein but containing only 1.4 to 1.6 per cent of P_2O_5. This I term, provisionally, Paranuclein A.

2. By acting at 40° C. on an acid, concentrated solution of the products of the peptic digestion of casein, containing no casein or paranuclein, with a concentrated solution of pepsin, a substance is precipitated which is identical in properties and in phosphorus content with the above-mentioned Paranuclein A.

3. The concentrated solutions of the casein products and of the pepsin, when kept separately at 40° C. give no precipitate, remain clear and homogeneous for a period of over three weeks, and at the end of that time yield no tests for paranuclein or for casein.

THE DETECTION AND ESTIMATION OF REDUCING SUGARS.

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THE DETECTION OF SUGARS.

Of the extremely large number of methods proposed for the detection of reducing sugars there are very few which may be regarded as specific for sugars alone. With one or two possible exceptions, these tests indicate only the presence or absence of reducing substances, and are inapplicable to the detection of sugars when other reducing substances are present. The fact that many of the sugars are powerful reducing agents in alkaline solutions, while they exert, at most, slight action in neutral or acid solutions, is very commonly recognized, and application is made of this fact in almost every test which has been proposed for the detection of these substances.

Gaud, Framm and others[1] have made studies of the effect of alkali upon a number of the sugars. The substances formed appear to be oxidation products, possibly preceded by a dehydration and decomposition. The destructive action of alkalies upon glucose is a common matter of reference throughout the literature upon the estimation of sugars, and numerous suggestions have been made to avoid this difficulty by means of the substitution of ammonium hydroxide for potassium or sodium hydroxide, and keeping the temperature somewhat below the boiling point during the reaction, with other devices of a similar nature.[2]

The following experiments were undertaken to throw light upon the mechanism of the reducing action of the sugars in alka-

[1] See De Bruyn and Ekenstein: *Rec. Trav. Chem.*, xvi, p. 274, 1897; Framm: *Arch. f. d. ges. Physiol.*, lxiv, p. 575, 1896, and Gaud: *Compt. rend. de l'Acad. des sci.*, cxix, p. 650, 1894.

[2] See Gaud: *loc. cit.*

line solution as well as to test the relative destructive action of certain of the alkalies upon various carbohydrates. A 1 per cent solution of dextrose was boiled for about a minute with one-half its volume of a 10 per cent potassium hydroxide solution, the resulting solution cooled to room temperature and added to an equal volume of ordinary Fehling's solution, also at room temperature. No change occurred in the mixture in the cold, and only a very faint reduction was obtained upon boiling. Clearly the sugar had been practically completely oxidized, so far at least as its power of affecting Fehling's solution was concerned. This result was rather what might have been expected, inasmuch as we are here dealing with one of the most readily oxidized of the carbohydrates.

In the next experiment a solution of lactose was substituted for the dextrose. Upon adding an equal volume of Fehling's solution to the cooled solution a heavy reduction of the copper occurred almost instantaneously *in the cold.*[1]

Five cubic centimeters of a 1 per cent solution of dextrose were now heated to boiling with about 0.5 gram of sodium carbonate, the resulting solution cooled, and mixed with an equal volume of Fehling's solution. An almost instantaneous reduction of the copper compound occurred, as indicated by a heavy precipita-

[1] The only references which I have been able to find relating to the formation of a compound capable of reducing Fehling's solution in the cold as a result of warming sugars with alkali, are in Kühne's *Lehrbuch der physiologischen Chemie* (p. 518, 1868), in the work of Worm Müller and Hagen (*Arch. f. d. ges. Physiol.*, xxii, p. 391, 1880), and of Emmerling and Loges (*Arch. f. d. ges. Physiol.*, xxiv, p. 184, 1881). Kühne states that dextrose solutions, heated to about 70° C. with sodium or potassium hydroxide solution will yield a solution capable of reducing Fehling's solution in the cold. He suggests this fact as evidence that the sugars themselves do not reduce directly and suggests this procedure as a test for dextrose in the urine. Worm Müller and Hagen discuss the bearing of this fact upon Trommer's test for dextrose in the urine. Emmerling and Loges come to the conclusion that the reducing substance formed is acetone alcohol, CH_3CO-CH_2OH. At the time my work was done the statement of Kühne on this subject was unknown to me, and inasmuch as neither he nor Worm Müller made comparisons of the action of the other alkalies and other sugars, nor gave a full theoretical discussion or analytical application of the fact which they stated, I have thought it desirable to give a rather complete discussion of my results.

tion of the yellow cuprous oxide. A solution of lactose substituted for the dextrose in the above experiment yielded a similar result. In other words, a 1 per cent solution of glucose boiled for a few moments with 10 per cent potassium hydroxide solution almost completely loses its reducing power. A similar treatment of a lactose solution yields a solution containing some substances capable of reducing Fehling's solution in the cold. Boiling either lactose or dextrose with sodium carbonate yields a solution which reduces Fehling's in the cold. It is particularly to be noted here that the potassium hydroxide solution destroys the reducing power of the dextrose, while the carbonate, under similar conditions, does not. This fact will be referred to later. The compound formed under these conditions is remarkably strong in its reducing power. Ammoniacal silver solutions are reduced instantaneously and completely in the cold. The product formed either from dextrose or lactose is capable of reducing Barfoed's reagent upon boiling, even when the acidity is considerably greater than that called for in Barfoed's formula.

These results indicate that the sugars themselves are not directly responsible for the strong reducing action of their alkaline solutions. They also admit of some valuable analytical applications. The first of such applications would be apparently to afford a differentiation of lactose from dextrose, the former substance in 1 per cent solution forming a compound upon boiling with alkali which will reduce Fehling's fluid in the cold, while dextrose under similar conditions is destroyed. This application can be made, and by means of such a method it is possible to detect lactose in presence of dextrose, or to distinguish between dextrose and lactose solutions. The procedure is as follows: To about 5–10 cubic centimeters of the solution to be tested is added one-half volume of 10 per cent potassium hydroxide solution and the resulting mixture boiled vigorously for about one and one-half minutes. The solution is then cooled and added to an equal volume of Fehling's fluid. The immediate formation of a heavy precipitate which is bright yellow and fills the entire body of the solution indicates lactose. A slight precipitate is to be disregarded. The solution used for this test should contain between 0.5 and 1 per cent of the sugar. It is not a particularly delicate reaction and will not give positive results where

the amount of lactose is under 25 per cent of the total quantity of sugar present.

A much wider analytical application of these facts is possible. Upon them we may base a reaction which is apparently specific for sugars. Very few, if any other substances will, upon boiling with alkalies, form substances capable of reducing Fehling's solution in the cold. In just so far as this is true, this method of detecting sugars is specific. The procedure is as follows: To 5–8 cubic centimeters of the fluid to be tested in a test tube, is added about one-half gram of sodium carbonate. Heat the solution to boiling and continue the boiling for about one-half minute. Cool and add an equal volume of Fehling's solution. If sugar is present a reduction will occur more or less promptly, one per cent or more giving a practically instantaneous reaction, smaller amounts requiring from three to five minutes. As thus carried out this method will detect sugar in solutions containing 0.1 per cent, or more, of sugar. Slightly warming the tube will increase the delicacy to about 0.05 per cent. These figures apply to relatively pure solutions of the sugars.

While this method is not nearly so sensitive as the usual Fehling's method, it may become of material use in establishing the presence or absence of sugars in mixtures containing other reducing substances, and is delicate enough for many purposes.

Attention was called above to the fact that sodium hydroxide solutions destroy the reducing properties of glucose much more rapidly than does sodium carbonate solution.[1] Yet the alkalinity given by sodium carbonate is amply sufficient for the glucose to exhibit its full reducing action. Considering the bearing of these facts upon sugar detection it will be evident that where only very small amounts of dextrose are present, or where some

[1] It is not to be inferred from this statement that a distinction is implied between the action of the hydroxides and carbonates in any respect other than may be accounted for by the difference in the degree of alkalinity represented by the two substances. If we use very dilute alkali solutions, stronger sugar solutions, or if the heating be more mild, it is perfectly possible, as Kühne and Worm Müller have shown, to obtain a solution which will still exhibit reducing properties. If, however, we endeavor to use hydroxide solutions sufficiently dilute to avoid the destruction of even minute quantities of dextrose, a solution is obtained which is utterly unfitted for ordinary qualitative or quantitative sugar work.

substance is present which temporarily interferes with the reduction of the copper solution, a reagent in which the alkalinity is secured by means of sodium carbonate, instead of hydroxide, should give more satisfactory results. In order to test this hypothesis the following work has been done.

Solutions of the following composition were prepared:

SOLUTION A.	
Crystallized copper sulfate	69.3 gm.
Distilled water to	1000 cc.
SOLUTION B.	
Pure Rochelle salt	346 gm.
Anhydrous sodium carbonate	200 "
Distilled water to	1000 cc.

For use, these solutions are mixed in equal proportions, and the resulting mixture diluted with an equal volume of distilled water. When further diluted with an equal volume of distilled water, heated to vigorous boiling and allowed to cool spontaneously, this solution should not show the slightest turbidity. The reagent thus prepared was tested regarding its power of detecting sugars as compared with Fehling's fluid, under the following conditions. When distilled water solutions of dextrose were used and the solution boiled as in the usual procedure, it was found possible to obtain in most cases a perceptible reaction with Fehling's fluid[1] when the sugar present amounted to 0.001 per cent. The result here was in many instances uncertain and marked the full limit of the test as I applied it. The method of procedure was to add to 3 cubic centimeters of Fehling's fluid in a test tube an equal volume of the solution to be tested, the resulting mixture being heated to vigorous boiling, which was continued for about one-half minute. The solution was then allowed to cool to room temperature, and unless precipitation had already occurred, it was again heated to boiling and allowed to cool, this process being sometimes repeated. Worm Müller and Hagen[2] state that Fehling's solution will indicate the presence of glucose in solutions containing 0.0008 per cent. I have been absolutely unable to obtain any reaction with 0.0008 per cent solutions of glucose with the procedure above described, while solutions of 0.001 per cent often gave negative results.

[1] The Fehling's fluid used was made up according to Soxhlet's formula.
[2] *Arch. f. d. ges. Physiol.*, xxii, p. 383, 1880.

With the reagent in which the carbonate is substituted for the hydroxide, according to the formula given above, and following an exactly similar method of procedure to that described for Fehling's solution, I have obtained absolutely positive results with solutions containing 0.00005 per cent of glucose. A distinct yellowish precipitate is obtained with glucose solutions of this dilution, especially upon cooling. Further dilutions were not tested. It thus appears that the substitution of carbonate for hydroxide yields a solution many times as delicate for the detection of sugars as we have in Fehling's fluid. A comparison made with any dilute solution—for instance, about 0.01 per cent solutions of dextrose—will speedily convince anyone of the greater merits of the carbonate reagent.

The urine, containing as it does substances which either partially reduce or else inhibit the reduction of Fehling's fluid, offers peculiar difficulties in the detection of small amounts of sugar. It is, therefore, of special interest to determine whether the carbonate solution possesses any advantages over Fehling's fluid for the detection of dextrose in the urine. Fehling's solution will usually indicate dextrose in the urine when present up to, or in excess of, 0.1 per cent. Smaller amounts give absolutely no indication of their presence, save possibly by confusing changes in the color of the solution. When the amount is as small as 0.1 per cent, the results are often very uncertain. With the reagent in which the carbonate is substituted for the hydroxide, it is perfectly possible to detect as small amounts of dextrose in the urine as from 0.015 to 0.02 per cent. Even these small quantities yield results which are almost invariably far more positive than can be obtained with Fehling's fluid in the presence of ten times the amount of sugar.

The procedure for the detection of sugar in the urine is as follows. Solutions A and B, made according to formulæ given above, are mixed in equal proportions. The resulting solution is then diluted by the addition of three times its volume of distilled water. To about 6 cc. of this reagent in a test tube are added from 7 to 9 (not more) drops of the suspected urine. The mixture is heated to vigorous boiling for about one-fourth to one-half minute, and allowed to cool spontaneously to room temperature. This process may be repeated if desired, though it is usu-

ally unnecessary. In the presence of sugar a precipitate will form which is often greenish or bluish green to begin with (in case the amount of sugar present is small), and usually becomes yellowish upon standing. This precipitate generally forms at or below the boiling temperature if the sugar present exceeds 0.06 per cent; with smaller amounts it forms slowly, usually only upon cooling. With larger amounts of sugar the reaction is obtained very readily upon reaching the boiling temperature. The precipitate is then generally reddish or yellowish in color. The results obtained in this test, even with the smaller amounts of sugar are extraordinarily definite, and according to my experience leave no room for uncertain interpretation. Normal urine will not, under these conditions, produce even the slightest turbidity in the reagent. Professor Mendel, of this laboratory, has suggested that the greenish precipitate obtained with urines containing small amounts of sugar, may be a compound of copper with the sugar, rather than a reduction product. While this may be the case, it seems more probable to the writer that the precipitate represents, if not a simple cuprous oxide or hydroxide, a compound of some constituent of the urine with reduced copper oxide. This appears the more likely, because, in the absence of the urine, sugar solutions of equal dilution give a definite reduction product of either the red oxide or the yellowish hydroxide. This latter compound, it may be remarked, is more usually obtained as a product of the reduction of the carbonate-copper solution, than is the case with Fehling's fluid, particularly when only small amounts of sugar are present, owing probably to the less strongly dehydrating action of the carbonate solution.

Whatever may be the nature of the precipitate obtained as a result of boiling the carbonate copper solution with sugar-containing urines, it appears to be a most delicate and satisfactory method for the detection of dextrose in this fluid.

It is interesting to note in this connection the modification of Fehling's test for sugar in the urine proposed by Worm Müller,[1] in which he suggests the use of solutions of sodium hydroxide more dilute than in ordinary Fehling's fluid, small amounts of copper solution, and the continued heating of the solution well

[1] Worm Müller: *Arch. f. d. ges. Physiol.*, xxvii, p. 107, 1882.

below the boiling point. This modification is capable of detecting about 0.03 per cent of dextrose in the urine. Worm Müller himself suggested that the superior delicacy of this test over the ordinary Fehling's method is due to the fact that the sugar reduces at a lower temperature than the reducing substances normally contained in the urine. In view of the facts brought out in the earlier portions of this paper it would be inferred that there are other factors here as well as the one he suggested. Thus it seems probable that ordinarily the interfering substances in the urine (chief among which is apparently creatinin, as pointed out by MacLean[1]) will inhibit the reducing action of the sugar long enough for the strong alkali to completely destroy the small amount of carbohydrate present. Lower temperature and weaker concentration of alkali would tend to prevent this destruction, so that eventually the glucose would have its normal reducing action. Similar results are apparently obtained where carbonate is substituted for the hydroxide. The procedure is not troublesome as in Worm Müller's test, and the results are more delicate and conclusive.

Thus we see that the carbonate solution has the following advantages over Fehling's solution. It is many times more delicate for the detection of sugars in pure solutions. This is just what we should expect from theoretical considerations. Fehling's fluid, containing as it does, a substance which is strongly destructive to glucose, should not be used so long as we can substitute something which is more effective and has not this destructive action. The carbonate solution yields more definite results than does Fehling's fluid, since there are fewer substances which interfere with its action than is the case with the other fluid. This can be shown by comparative tests with the urine, and further it can be most beautifully exemplified by comparisons of the reducing action of chloroform upon the two solutions. A solution of chloroform in water will reduce Fehling's solution most copiously, even slightly below the boiling point, whereas its action is very slight upon the carbonate solution and only occurs upon prolonged boiling. There are many other substances which reduce Fehling's solution more readily than the carbonate

[1] MacLean: *Bio-chem. Journ.*, i, p. 111, 1906.

solution, and thus interfere with the use of the former solution as a test for sugars to a greater degree than is the case with the carbonate solution; but chloroform is the most striking example. Regarding the stability of the carbonate solution the following should be stated. For delicate work in sugar detection, either in pure solutions or in the urine, the solutions making up the reagent should be freshly mixed and diluted. If it is desired to keep the mixed reagent on hand the two solutions should be mixed in equal proportions and to every liter of the undiluted mixture should be added from five to ten grams of sodium hydroxide. This small amount of alkali serves to prevent decomposition and does not affect the delicacy of the reagent to any great extent. Such a mixture will remain for weeks more delicate as a reagent than Fehling's solution and is not nearly so caustic. It should be remembered, however, that the best results are only above obtained with freshly mixed solutions diluted according to the directions.

A few copper solutions containing carbonates have been proposed in the literature. The best known of these is Soldaini's solution,[1] which contains 3.464 grams of crystallized copper sulfate and 297 grams of potassium bicarbonate dissolved in a liter of water. Ost[2] later modified this solution by substituting for a portion of the bicarbonate, a nearly equal weight of the normal carbonate, as well as increasing the amount of copper sulfate present. These solutions are open to serious objections to which the solution suggested in this paper is not. Soldaini's solution is more difficult to prepare, a portion at least of the copper always being left undissolved as the carbonate, which must be filtered off. Furthermore the boiling with the test solution must be continued for some time, often continuously for ten or fifteen minutes before smaller amounts of sugars indicate their presence by even the slightest apparent reduction. The reason for this fact appears to be that bicarbonate is capable of holding considerable amounts of cuprous oxide in solution, or else of preventing the reduction of cupric compounds by dextrose. This is readily proved by the following experiment. If to a portion of

[1] Soldaini: *Chem. Centralbl.*, p. 389, 1889.
[2] Ost: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, 1, p. 1035, 1890.

the carbonate copper solution described earlier in this paper a considerable amount of bicarbonate of sodium is added, it will be found that the resulting solution shows no reduction even with large amounts of sugar except upon very long continued boiling, while a similar solution to which the bicarbonate has not been added gives a heavy precipitate even before the boiling point is reached. Thus it will be seen that the bicarbonate solutions are open to marked objection in the fact that they require very prolonged boiling to obtain any result, and are, therefore, scarcely applicable to qualitative work. Results are obtained with the solution above recommended almost instantaneously, unless the amount of sugar present is very small, in which case it may require from four to five minutes.

A METHOD FOR THE VOLUMETRIC ESTIMATION OF SUGARS.

Owing to the depth of color of the precipitate obtained upon the reduction of Fehling's solution by means of sugars, the application of Fehling's process to the volumetric determination of sugar solutions is very difficult Even with long practice it is almost impossible to obtain satisfactory results by the use of this method. Various modifications of the original Fehling's fluid have been proposed with a view to render it more applicable to volumetric work. Most of these are based upon an attempt to keep the cuprous oxide formed in solution. In Pavy's solution ammonia is added for this purpose. The objections to the latter method are the great ease with which the dissolved cuprous compound undergoes oxidation—hence the absolute necessity of entirely excluding air if accurate results are to be obtained—and furthermore the rapidity with which the ammonia boils out of the solution. In Gerrard's method potassium cyanide is used as the solvent for the reduced copper compound. As ordinarily performed this method requires two titrations and must be carried out quite rapidly in order to avoid reoxidation of the reduced copper. The potassium cyanide solution used is unpleasant to work with and is quite unstable.

To the writer it seemed that a more satisfactory method of solving the difficulties offered by Fehling's solution in sugar titration would be to obtain some modification of the solution, which should yield upon reduction not the red cuprous oxide but some

colorless insoluble compound. In this case we should have the reduced copper compound in such a form as would prevent its reoxidation, while it would not obscure the end point of the reaction.

The well known insolubility of cuprous compounds of the halogens led to the attempt to obtain the precipitation of the reduced copper as one of the haloid salts, through the addition of considerable quantities of chlorides, bromides, or iodides to Fehling's solution. No satisfactory results were obtained with these substances. Closely related in chemical behavior to the halogens are the simple and complex cyanides, and the attempt was next made to see whether any of these substances would yield the desired results. Addition of potassium ferrocyanide to Fehling's solution causes, upon reduction, the precipitation of a white compound, which, however, upon continued boiling becomes dark in color. This substance was, therefore, unsatisfactory except in a certain combination referred to later. If potassium sulfocyanide be added, in small amounts, to Fehling's fluid it produces no appreciable change in the reduction product. If, however, it be added in considerable excess, the cuprous oxide formed will be held in solution. After the writer had found that carbonate copper solutions are much less destructive in their action upon dextrose than is the corresponding hydroxide solution, as has been shown earlier in this paper, it was only natural to try the above suggested compounds in connection with this solution. It was speedily found that in this case potassium sulfocyanide yielded a very different result from that obtained with Fehling's solution. After addition of potassium sulfocyanide to the carbonate solution it was found that upon reduction a chalk-white compound of cuprous sulfocyanide was produced, instead of the red oxide. This suggested at once the employment of this solution for volumetric purposes.

This fact was discovered by the writer early in November, 1906. A somewhat hasty review of the literature at that time, preliminary to working up the method, failed to reveal the fact that potassium sulfocyanide had ever been previously applied to sugar estimation, or even that the fact above stated had been mentioned in the literature. About January 1, 1907, a paper

appeared by Bang,[1] describing a volumetric method for sugar estimation, depending upon the use of potassium sulfocyanide. In this paper Bang referred to the fact that he had pointed out in an earlier communication[2] that addition of potassium sulfocyanide to alkaline copper solution which contained no hydroxide, caused the precipitation of white cuprous sulfocyanide instead of the red suboxide. He had also suggested in this earlier paper a method for the estimation of dextrose, based upon this fact. Bang's earlier paper had completely escaped my attention in the review of the literature on this subject, owing very probably to the fact that its title was in nowise concerned with sugar analysis, being "Ueber die Verwendung der Zentrifuge in der Quantitativen Analyse." The first method Bang proposed was based upon an attempt to estimate the excess of potassium sulfocyanide remaining in the solution after the reduction of the copper had taken place. This method appears to the writer obviously unsatisfactory. The cuprous sulfocyanide formed is not completely insoluble in even a small excess of potassium sulfocyanide, and unless some excess of this salt is present a portion of the copper is precipitated as the suboxide. It would thus appear that determination of the residual potassium sulfocyanide would not yield very satisfactory results. Bang recognized the unsatisfactory nature of his earlier method and in his second paper substitutes one which he considers much better. His method in this case, briefly outlined, is as follows: The copper solution employed is a modified Soldaini's solution, to every liter of which is added two hundred grams of potassium sulfocyanide. To a measured volume of this solution is added a measured volume of the sugar solution to be determined, the mixture then being boiled for three minutes. (No precipitation takes place owing to the large excess of sulfocyanide present.) Then the solution is cooled and the excess of cupric copper determined by titration with standard hydroxylamine solution to a colorless solution. The results obtained appear to be very satisfactory. There are at least two objections to Bang's method. The boiling copper solution cannot be titrated with the sugar solution directly to

[1] *Biochem. Zeitschr.*, ii, p. 271, 1906 (December).

[2] *Festschrift für Hammarsten*, 1906 (Upsala Läkareförenings Forhandlingar. Ny Foljd, Elfte Bandet. Supplement).

a colorless solution because of the employment of the bicarbonate solution of Soldaini and Ost. This solution, as is mentioned earlier in this paper, requires continued boiling before complete reduction takes place, which renders impracticable any attempt to titrate directly to an end point. The second objection to Bang's method is the employment of hydroxylamine for his final titration, a substance which even in form of its salts, is unstable, and not commonly available.

In the use of the writer's method for the volumetric estimation of sugar three solutions are required. which are made up according to the following formulæ:

SOLUTION A.	
Crystallized copper sulfate	69.30 gms.
Distilled water to	1000 cc.
SOLUTION B.	
Crystallized Rochelle salt	346 gms.
Pure anhydrous sodium carbonate	200 "
Distilled water to	1000 cc.
SOLUTION C.	
Potassium sulfocyanide	200 gms.
Distilled water to	1000 cc.

For use these solutions are mixed in equal proportions in the order indicated. To every 30 cubic centimeters of the solution thus obtained are added from 2.5 to 5 grams of pure anhydrous sodium carbonate.[1] The amount of this substance added should roughly correspond to the dilution to which the solution will be

[1] Regarding the addition of sodium carbonate in this connection the following should be stated. The alkalinity secured by the addition of the indicated portion of Solution B, is not great enough to give the most satisfactory end point. This lack of alkalinity might be overcome either by the addition of the solid carbonate as above suggested, or through the substitution of a greater amount of potassium carbonate in the formula of Solution B. (Solution B is practically saturated with sodium carbonate, which is, it will be remembered, much less soluble than the corresponding potassium compound.) I prefer the method above detailed for securing the increased alkalinity. Sodium carbonate is much commoner than the potassium salt, keeps anhydrous better and is usually more available. The addition of a small portion of the dry salt, which may be roughly measured, is certainly no great trouble. Furthermore, the formula of Solution B, it will be noticed, coincides with the one found most satisfactory for qualitative work, as detailed earlier in this paper, so that such a solution is readily available for either qualitative or quantitative work.

subjected during the titration, *i. e.*, for titrating dilute sugar solutions add greater quantity of carbonate and vice versa. The solutions are mixed in a beaker of suitable capacity, the requisite quantity of carbonate added, and the mixture heated to boiling over a gauze until the carbonate completely dissolves. Thirty cubic centimeters of this mixture (equivalent to 10 cubic centimeters copper sulfate solution) are equal to approximately 0.073 gram of pure dextrose.

The titration is carried out as follows—the sugar solution is run in from a burette rather rapidly—(not so rapidly as to interfere markedly with continuous vigorous boiling) until a heavy, chalk-white precipitate is formed and the color of the fluid begins to lessen perceptibly. The last portions should be run in in quantities of from two to ten drops (depending on depth of color remaining and the relative strength of the sugar solution), with a vigorous boiling of about one-fourth minute between each addition. The end point of the reaction is the complete disappearance of the blue color. This point is sharp and satisfactory. The precipitate obtained is chalk-white and is rather an aid than a hindrance to the determination of the end point. While potassium sulfocyanide could be added in large enough excess to retain the precipitate in solution, I can see no advantage in such a procedure, whereas it has the disadvantage of permitting some reoxidation if the titration be carried on too slowly.

It may be of interest to mention a simple devise used by the writer with much success to prevent the annoying bumping of the solutions during the process of titration. This consists in the introduction into the titration beaker of a medium sized piece of pure, previously well washed, absorbent cotton. By stirring this cotton about as the titration proceeds, it is possible to entirely prevent the bumping which otherwise may become very troublesome. Glass wool may be used in place of cotton but is not much more satisfactory and is considerably less economical.

In order to test the accuracy and reliability of this method a solution of dextrose in distilled water was prepared of approximately one per cent. The sugar content of this solution was determined by Allihn's gravimetric method, duplicate analyses being made in every case. Using this solution, the value of the copper solution used in the above method was computed from

the results of three titrations, in which were employed 10, 20 and 30 cubic centimeters of the copper sulfate solution, respectively. The results of these titrations were exactly concordant, giving the value of one cubic centimeter of copper solution as equivalent to .00727 gram of dextrose. On the basis of this standardization the strength of about twenty-five sugar solutions was determined, duplicate titrations being made in every case. The actual amount of sugar present in each solution was also determined by Allihn's gravimetric method and the results thus obtained compared with the results of the titrations. Where the solution titrated was too strong for determination directly by Allihn's method, it was diluted with a measured volume of water and then analyzed.

Duplicate analyses were invariably entirely concordant, the results differing either not at all or only by such slight error as is experienced in reading the ordinary burettes. The titrated solutions varied in strength from 2.2 to 0.1 per cent of dextrose. To some of the solutions were added small portions of the common inorganic salts, such as might occur as impurities in ordinary sugar solutions. The results were not affected by the presence of these substances.

The values obtained for the various solutions were in every case in very close agreement with the results found by Allihn's method. So far as could be determined, the value of the copper solution in terms of dextrose remained absolutely constant for strong or dilute solutions. In fact, in the case of many of the dilute solutions, *i. e.*, under 0.3 per cent, the results were more closely in agreement with the actual sugar content (known in these cases because obtained by exact dilution of stronger solutions) than could be obtained by Allihn's method, which, while giving very exact results and duplicates with solutions of about five-tenths of one per cent, is not so satisfactory for more dilute, or stronger solutions.

Attention is called to the fact that the value of the copper solution remains the same for dilute as for stronger solutions. This is not the case with many of the solutions employing the alkali hydroxides. I believe this fact finds ready explanation in the less strongly destructive action of the carbonates than the hydroxides, as pointed out earlier in this paper.

A table is appended giving results of five typical titrations, carried out in exact accordance with the directions given above.

No.	Grams of Glucose in 10 cc. of Solution.			
	By Allihn's Method.	Average from Allihn's Method.	Writer's Titration Method.	Average of Results by Writer's Method.
1	0.0280 0.0260	0.0270	0.0280 0.0274	0.0277
2	0.0530 0.0531	0.0530	0.0528 0.0528	0.0528
3	0.1021 0.1012	0.1016	0.1020 0.1022	0.1021
4	0.1810 0.1819	0.1814	0.1815 0.1820	0.1817
5	0.0112 0.0102	0.0107	0.0117 0.0113	0.0115

It should be stated here that where the sugar solution to be titrated contains less than three-tenths of one per cent, the writer has found the following method more expeditious and satisfactory, as avoiding extreme dilution during the titration. A measured amount (25 cc.) of the sugar solution to be determined is run into 30 or 60 cubic centimeters of the copper titration solution, the mixture boiled for from five to seven minutes and the residual copper determined with a dextrose solution of known strength, of approximately 1 per cent. While not an essential procedure, this method is expeditious and yields accurate results. In ordinary titrations the writer has used the mixed fluid in quantities of 30 or 60 cubic centimeters, the former volume being sufficient for sugar solutions of 1 per cent or less.

In cases where it is not convenient to standardize the copper solution against sugar solutions, of known strength, it is possible to obtain quite satisfactory results through the employment of an exact weight of pure crystallized copper sulfate. Where this method is employed the copper sulfate should be freshly recrystallized, dried upon blotting or filter paper and accurately weighed out upon an analytical balance, 69.30 grams being dissolved in water and the volume made up to exactly one liter. Ten cubic centimeters of this solution are equivalent to approx-

imately .073 gram of dextrose. This process was tried upon three occasions by the writer and the results varied but slightly from those obtained through standardization of the solution with sugar solutions of known strength, though for absolute accuracy the latter process is of course to be preferred.

Some titrations have been made to test the applicability of this method to the determination of dextrose in the urine.[1] The results obtained show a quite satisfactory approximation to the actual sugar content, although, just as should be expected, they are not exact, the variation usually being from five-hundredths to two-tenths of one per cent of the actual sugar content, depending upon the relative amount of dextrose present. Solutions containing larger percentages of sugar yield more correct results.

In conclusion it may be stated that the writer has met with certain substances (notably traces of chloroform) which are capable of causing a portion of the copper to precipitate as the red oxide, even in presence of the sulfocyanide. While impurities which will do this would be only rarely encountered, and although the interference with the end point is usually not marked, it is desired to offer an alternative formula for Solution C, which will obviate this slight difficulty entirely. The formula for this solution is as follows:

Potassium ferrocyanide	30 gms.
Potassium sulfocyanide	125 "
Anhydrous sodium carbonate	100 "
Distilled water to	1000 cc.

The use of this solution does not change the value of the copper in terms of dextrose, and may be used entirely in place of the other formula if desired.

This method has not yet been tested regarding the other reducing sugars except to find that they also yield the white precipitate as a result of their action on the solution. There is no apparent reason why there should be any difficulty in such an application.

[1] Rudisch and Celler (*Journ. Am. Med. Assoc.*, Jan. 26, 1907, p. 324) describe a method for determination of sugar in the urine through the use of ordinary Fehling's fluid to which is added a large amount of potassium sulfocyanide, thus preventing precipitation of cuprous oxide when reduction occurs.

THE HEAT OF COMBUSTION OF VEGETABLE PROTEINS.

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Very few determinations of the heat of combustion of vegetable proteins are to be found in the literature. The earliest appear to be those made by Danilewsky,[1] who found for "Pflanzen-fibrin" 6231, for legumin 5573 and gluten 6141 calories per gram.

As these determinations were made according to Stohmann's method at a time when this method was not perfected and as numerous precautions, which later were found to be important, were not considered, Stohmann[2] made new determinations of the heat of combustion of several proteins, among which were the crystallized globulin of the squash seed, prepared by Grübler, for which he found 5598 calories, and conglutin, which gave 5362 calories per gram.

Berthelot and Andre,[3] using the Berthelot bomb found for "Vegetable fibrin" 5837, for crude gluten 5995 calories per gram.

Stohmann and Langbein,[4] next determined the heat of combustion of various carefully prepared proteins using the calorimetric bomb of Berthelot. Most of the preparations were made by Grübler and before combustion were protractedly extracted with ether. The substances were burned in the air dry state and the results calculated to a water and ash-free basis. They found for "Pflanzen-fibrin" (glutenin of wheat) 5942, for legumin from white beans (phaseolin) 5793, for the crystallized globulin of squash seed 5672, and for conglutin from lupines 5479 calories per gram. No other determinations of the heat of combustion of vegetable proteins are known to us.

[1] Danilewski: *Centralbl. f. d. med. Wissensch.*, xix, pp. 465 and 486, 1881.
[2] Stohmann: *Journ. f. prakt. Chem.*, xxxi, p. 273, 1885.
[3] Berthelot and André: *Ann. de chim. et de phys.*, xxii, p. 25, 1891.
[4] Stohmann and Langbein: *Journ. f. prakt. Chem.*, xliv, p. 336, 1891.

In view of the very small number of vegetable proteins of which the heat of combustion has been determined and of their importance in so much of the food consumed both by men and animals, it seemed important to determine this factor for a large number of these substances derived from many of the different kinds of seeds in general use as food.

These proteins were all prepared with special reference not only to their separation from all non-protein bodies but also from other associated proteins. In other words, the preparations represent as nearly as possible definite chemical individuals. That they are in fact definite chemical individuals we are unable to assert for no method is yet available whereby this fact can be established. These preparations, however, represent substances that we have been unable to separate by fractionation into different parts, the composition or properties of which would indicate a mixture. They must for the present be accepted as representing the most definite protein products that can be prepared from the seeds that yielded them. These substances were burned in the Berthelot-Atwater calorimetric bomb.

The calorimeter room was kept at the most constant temperature possible and all the minor precautions, suggested by experience with over 10,000 of these combustions, were employed.

The bomb used in these determinations was so adjusted, as regards its hydrothermal equivalent, as to give the heat of combustion of pure, anhydrous cane sugar as 3959 calories per gram and of pure, fused benzoic acid as 6322 calories per gram.

From 0.5 to 0.8 gram of substance was used for each combustion which was weighed after the thoroughly dried material had assumed constant weight by prolonged exposure to the air.

As it is of the utmost importance that a definite state of desiccation should be established to which the heat of combustion should be referred a series of preliminary experiments were tried with the globulin edestin. The preparation of edestin, made according to the method described by Osborne,[1] was neutral to phenolphthalein and had been recrystallized several times.

Of this preparation 16 samples of the air dry material, each weighing 2 gms. were dried in a high vacuum for 9 days, 17 days, 24 days and 46

[1] Osborne: *Journ. of the Amer. Chem. Soc.*, xxiv, p. 39, 1902; also *Zeitschr. f. physiol. Chem.*, xxxiii, p. 240, 1901.

days and weighed at the end of each period. The average percentage of water, as determined by the total loss of weight at the end of each period, was 5.64, 5.76, 5.95, and 5.99, respectively, the agreement between the several samples being remarkably close. The average weight of material in each dish was therefore 1.8802 gram. After the desiccated edestin had been exposed to the air of the room the average weight in each dish was 1.9911 gram, the protein having regained very nearly all the moisture lost in the desiccator.

The heat of combustion of this material was determined with the following results:

	5184	calories per gram.
	5202	" "
	5185	" "
	5199	" "
	5196	" "
	5193	" "
	5194	" "
Average,	5193	" "

Calculating this result to an ash- and water-free basis, as determined by the above desiccation, we have 5507 calories per gram of edestin.

Of the same edestin 6 samples of 2 gms. each were weighed in shallow aluminum dishes, provided with a tight fitting cover,[1] and dried at 110° for successive periods of 5, 3½ and 4 hours, weighed after cooling in a desiccator, and then placed in the high vacuum for 5 weeks. At the end of this time the loss of weight was 8.37 per cent. After standing for some time in the room, exposed to the air, 4 samples were burned with results shown below:

Dry Weight.	Weight after Standing.	Heat of Combustion, Calories per Gram.	Heat of Combustion Calculated to an Ash- and Water-free Basis.
1.8353 gm.	1.9685 gm.	5228	5614
1.8353 "	1.9700 "	5247	5639
1.8326 "	1.9726 "	5226	5632
1.8322 "	1.9741 "	5222	5633
		Average,	5629

A comparison of these moisture determinations shows that while the highest per cent of moisture found by desiccation at the room temperature in a very high vacuum was 5.99 that found by heating at 110° C. and subsequently drying in the vacuum desiccator for a long time was 8.37 per cent or 2.38 per cent more.

While this large difference in the apparent water content was found in this sample of edestin, in three other samples of edestin the difference was found to be but 0.08, 0.42, and 0.39 per cent,

[1] Cf. Benedict and Manning: *Amer. Journ. of Physiol.*, xiii, p. 309, 1905; xviii, p. 309, 1907.

respectively. We are at present unable to explain the marked difference in behavior of these samples of edestin. In a large number of other vegetable proteins the difference was found to be not far from 0.25 per cent.

It has previously been shown by one of us[1] that protein preparations which have been dried by very long desiccation in a high vacuum over sulphuric acid show a gain in weight when subsequently dried at 110° C. in air. This gain in weight is more than lost if the protein after drying at 110° is again dried for a sufficient time *in vacuo* over sulphuric acid.

The fact that heating at 110° C. and subsequently drying in a vacuum results in a slightly larger loss of weight indicates a slight loss of water of constitution from the protein, which water, owing to the very high hygroscopic power of the dry protein, is retained as moisture, even at 110°, and is subsequently lost in the vacuum desiccator. That this explanation is correct is, however, rendered doubtful by the results obtained in drying the preparations for the determinations of the heats of combustion that are later described in this paper. A certain number of these preparations had, before using them for these experiments, been dried at 110°, the others dried only over sulphuric acid at the room temperature. The results, however, in most cases showed practically the same differences in moisture as determined by the two methods of drying just described. It would seem probable that if this difference was due to water of constitution lost at 110° these samples which had previously been dried at 110° would show no difference in the result of drying by the two methods, for it has been our experience that the protein preparations assume constant weight after drying for a few hours at 110°.

This excess of moisture, found after drying at 110° and then *in vacuo*, over that found by drying *in vacuo* alone, is shown by the following table:

Preparation Previously Dried over Sulphuric Acid.		Preparation Previously Dried at 110° C.	
Conglutin	0.18 per cent.	Amandin	0.28 per cent
Edestin No. 1	0.08 "	Excelsin	0.29 "
" No. 2	0.42 "	Vignin	0.76 "
" No. 3	0.39 "	Glycinin I	0.29 "
Cotton-seed globulin	0.28 "	Legumin, lentil	0.06 "

[1] Cf. Benedict and Manning: *Amer. Journ. of Physiol.*, xviii, p. 213, 1907.

Preparation Previously Dried over Sulphuric Acid.			Preparation Previously Dried at 110° C.		
Phaseolin, adzuki bean	0.21	per cent.	Legumin, horse bean	0.08	per cent.
Hordein	0.20	"	Legumin, Vetch	0.20	"
Edestin 40	2.38	"	Phaseolin, adzuki bean	0.13	"
Conglutin β	1.26	"	Phaseolin, kidney bean	0.20	"
			Conglutin α	0.07	"
			Glutenin	0.09	"
			Glycinin II	0.25	"
			Edestin No. 4	0.68	"

With the exception of Edestin No. 40 and Conglutin β the preparations that had previously *not* been heated show about the same differences as those that had already once been dried at 110° and it would therefore appear that this difference is, in most cases, due to some undetected condition of manipulation. No explanation of the large difference shown by Edestin No. 40 is apparent. This might be attributed to water of crystallization were it not for the fact that the other samples of edestin were likewise crystalline.

In order to detect any possible effect of heating during the drying a large number of determinations of the heat of combustion were made on material that had been dried *only* at room temperature *in vacuo* and the heat of combustion calculated to an ash- and moisture-free basis, the moisture being determined solely *in vacuo* and for comparison with these the heat of combustion was also determined in duplicate samples that had been dried at 110° and then for a long time *in vacuo*, as just stated. In a few samples the moisture was determined simply by long drying in the vacuum and the results in these cases are probably stated a small fraction of 1 per cent too low.

Returning now to the sample, Edestin No. 40, it is to be noted that the heat of combustion of the sample which had been dried *in vacuo* was 5507 calories per gram, calculated to an ash- and water-free basis as determined by drying *in vacuo*, whereas the heat of combustion of the sample dried at 110° and afterwards *in vacuo* was 5629 calories per gram, calculated to the ash- and water-free basis as determined by drying at 110° and then *in*

vacuo. If the first determination of the heat of combustion is recalculated on the assumption that the difference in water determinations by the two methods is due to moisture still present in the sample dried only *in vacuo* we find the heat of combustion to be 5630, which agrees exactly with the second determination, 5629 calories per gram. In confirmation of this result four other samples of edestin were dried in the two ways and the following results obtained, calculated to an ash- and water-free basis; *a*, moisture determined *in vacuo; b*, moisture determined by heating at 110° for 12½ hours and then drying in vacuum desiccator for 5 weeks. The result given under *a* is calculated under *c* on the assumption that the difference in moisture found between *a* and *b* was due to moisture still retained in the samples dried by method *a*.

	a	*b*	*c*	
Edestin No. 40	5500	5622	5623	calories per gram.
" " 1	5570	5658	5575	" "
" " 2	5575	5655	5600	" "
" " 3	5629	5668	5653	" "
" " 4	....	5644	....	" "

The difference in the moisture determinations in samples Nos. 1, 2 and 3, dried by the two methods, is much less than that found for Edestin No. 40 and the difference in the heats of combustion is correspondingly less.

In the following tables we give the results of the determinations of the heats of combustions of a large number of different vegetable proteins. The condition of these preparations as respects drying before using in this work is indicated in Column I by "dried at 110°" or "over H_2SO_4" respectively. Under VII is given the heat of combustion as found for the preparation dried only *in vacuo* calculated to a water-free basis, assuming that the slightly greater loss of weight after drying at 110° and then *in vacuo* is moisture.

AMANDIN. This is a globulin forming the greater part of the protein matter of the seeds of the almond (*Prunus amygdalus var. dulcis*).

Dried at 110° in air it has the following composition:[1]

C, 51.30; H, 6.90; N, 18.90; S, 0.43; O, 22.47 per cent.

[1] The composition of all these proteins was determined after drying in the customary way at 110° in air without subsequent drying in vacuo.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
at 110°	in vacuo	1.8063 gm. 1.8054 "	1.9787 gm. 1.9784 "	5037 5022	5527	5543
	at 110° and then in vacuo	1.8001 " 1.8002 "	1.9822 " 1.9820 "	5022 5018	5543	

CORYLIN. This globulin constitutes most of the protein substance of the filbert or hazel-nut (*Corylus avellana*). Its composition, dried in air at 110°, is

C, 50.72; H, 6.86; N, 19.03; S, 0.83; O, 22.56 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
over H_2SO_4	in vacuo	1.8237 gm. 1.8238 "	1.9980 gm. 2.0009 "	5036 5053	5573	5583
	at 110° and then in vacuo	1.8202 "	2.0046 "	5044 5044	5597	

EXCELSIN. Most of the protein of the Brazil or Para nut (*Bertholletia excelsa*) consists of the globulin excelsin which crystallizes in hexagonal plates. The preparation used for these experiments consisted entirely of hexagonal plates. Its composition is

C, 52.23; H, 6.95; N, 18.26; S, 1.09; O, 21.47 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
at 110°	in vacuo	1.8552 gm. 1.8552 "	2.0280 gm. 2.0271 "	5224 5212	5719	5737
	at 110° and then in vacuo	1.8500 " 1.8485 "	2.0312 " 2.0307 "	5197 5222	5736	

EDESTIN. This is the principal protein constituent of the hemp-seed

(*Cannabis Sativa*). The preparations used for these combustions were all composed of octahedral crystals. The composition of Edestin is

C, 51.36; H, 7.01; N, 18.65; S, 0.88; O, 22.10 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
Prep. No. 1 over H_2SO_4	in vacuo	0.8980 gm. 0.8980 "	0.9960 gm. 0.9961 "	5006 5008	5570	5575
	at 110° and then in vacuo	0.8972 " 0.8972 "	0.9867 " 0.9867 "	5126 5133	5658	
Prep. No. 2 over H_2SO_4	in vacuo	0.9092 " 0.9087 "	1.0083 " 1.0081 "	5018 5020	5575	5583
	at 110° and then in vacuo	0.9072 " 0.9024 "	0.9989 " 0.9925 "	5113 5165	5630 5681	
Prep. No. 3 over H_2SO_4	in vacuo	0.9098 " 0.9102 "	1.0289 " 1.0296 "	4973 4971	5629	5653
	at 110° and then in vacuo	0.9045 " 0.9077 "	0.9977 " 0.9985 "	5146 5136 5156	5673	
Prep. No. 4 at 110°	in vacuo	0.9182 " 0.9114 "	1.0086 " 0.9966 "	5138 5135 5135	5644	

Globulin of the Cotton Seed (*Gossypium herbacium*). Very nearly all of the protein matter of this seed consists of this globulin, the composition of which is

C, 51.71; H, 6.86; N, 18.30; S, 0.62; O, 22.51 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
over H_2SO_4	in vacuo	1.8179 gm. 1.8181 "	1.9728 gm. 1.9741 "	5179 5113 5258	5657	5673
	at 110° and then in vacuo	1.8132 " 0.9054 "	1.9939 " 0.9966 "	5074 5059 5074 5044 5046	5596	

VIGNIN. The greater part of the protein substance of the cow pea (*Vigna sinensis*) consists of this protein which has the properties of a globulin and the following composition:

C, 52.64; H, 6.95; N, 17.25; S, 0.42; O, 22.74 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
at 110°	in vacuo	0.9206 gm. 0.9182 "	1.0292 gm. 1.0268 "	5055 5039	5668	5715
	at 110° and then in vacuo	0.9118 "	0.9944 "	5202 5210	5703	
over H_2SO_4	in vacuo	0.9081 " 0.9071 "	1.0242 " 1.0240 "	5023 5027	5688	
	at 110°* and then in vacuo	0.4552 " 0.4551 "	0.4991 " 0.4999 "	5160 5178	5691	5721

* This was the only preparation which showed a greater loss of weight after drying in vacuo alone than after drying at 110° and then in vacuo. No reason for this exception is apparent. Were it not for the fact that the heats of combustion of the two samples agreed so closely it might be taken as indicating oxidation.

GLYCININ. This globulin forms the greater part of the protein matter of the soy bean (*Glycine soja*). Its composition is

C, 52.01; H, 6.89; N, 17.47; S, 0.71; O, 22.92 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
yellow soy bean at 110°	in vacuo	1.8242 gm. 1.8254 "	1.9620 gm. 1.9655 "	5236 5242 5255	5667	5687
	at 110° and then in vacuo	1.8181 " 1.8197 "	1.9938 " 1.9979 "	5147 5147	5672	
Japanese soy bean over H_2SO_4	in vacuo	0.9184 " 0.9187 "	1.0248 " 1.0247 "	5056 5016	5665 5619	5680 5636
	at 110° and then in vacuo	0.9170 " 0.9151 "	1.0236 " 1.0211 "	5048 5048	5657	

LEGUMIN. Most of the protein matter of seeds of the horse bean (*Vicia faba*), lentil (*Ervum lens*), vetch (*Vicia sativa*), and pea (*Pisum sativum*) consists of a globulin, the preparations of which have thus far appeared to be identical in respect to all their properties that have been examined. This agreement is now found for the heats of combustion of preparations from the three seeds first named. No preparation from the pea was available at the time this work was done. The composition of legumin is

C, 51.72; H, 6.95; N, 18.04; S, 0.39; O, 22.90 per cent.

Legumin, lentil.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
at 110°	in vacuo	0.9094 gm. 0.9092 "	0.9771 gm. 0.9769 "	5224 5203 5202	5612	5619
	at 110° and then in vacuo	0.9082 " 0.9093 "	0.9835 " 0.9850 "	5177 5193	5630	

Legumin, horse bean.

at 110°	in vacuo	0.9122 " 0.9112 "	0.9820 " 0.9826 "	5203 5190	5625	5632
	at 110° and then in vacuo	0.9112 " 0.9107 "	0.9872 " 0.9869 "	5220 5172	5656	

Legumin, vetch.

at 110°	in vacuo	0.9088 " 0.9092 "	0.9830 " 0.9840 "	5156 5143 5143	5586	5600
	at 110° and then in vacuo	0.9063 " 0.9077 "	0.9878 " 0.9865 "	5132 5150 5166	5621	

PHASEOLIN. This is a globulin that forms the principal part of the protein substance of the white or kidney bean (*Phaseolus vulgaris*), and also that of the Japanese adzuki bean (*Phaseolus radiatus*). Its composition is

C, 52.57; H, 6.97; N, 15.84; S. 0.33; O, 24.29 per cent.

Phaseolin, kidney bean.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
at 110°	in vacuo	1.8270 1.8276	2.0409 2.0403	5050 5049	5675	5689
	at 110° and then in vacuo	(1.8232	2.0273	5083 5060 5086	5679	

Phaseolin, Adzuki bean.

at 110°	in vacuo	0.9226 gm. 0.9237 "	1.0213 gm. 1.0227 "	5123 5111	5709	5726
	at 110° and then in vacuo	0.9213 " 0.9225 "	1.0202 " 1.0216 "	5095 5088	5683	
over H_2SO_4	in vacuo	0.9061 " 0.9066 "	1.0070 " 1.0072 "	5072 5081	5681	5693
	at 110° and then in vacuo	0.9045 " 0.9040 "	1.0029 " 1.0030 "	5097 5112	5702	

CONGLUTIN. The substance formerly called conglutin, which forms almost all of the protein matter of the seeds of lupines (*Lupinus*) of various species consists in those of the yellow lupine (*Lupinus luteus*) of at least two distinct globulins. These are designated Conglutin α and Conglutin β. In the seeds of blue lupine (*Lupinus angustifolius*) the conglutin is similar to Conglutin α. The composition of conglutin from the blue lupine is

C, 51.13; H, 6.86; N, 18.11; S, 0.32; O, 23.10 per cent.

Conglutin α has a similar composition, namely,

C, 51.75; H, 6.96; N, 17.57; S, 0.62; O, 23.10 per cent.

Conglutin β has the following composition[1]

C, 49.91; H, 6.81; N, 18.40; S, 1.67; O, 23.21 per cent.

[1] Cf. Osborne and Harris: *Amer. Journ. of Physiol.*, xiii, p. 436, 1905.

Conglutin, Blue lupine.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
over H_2SO_4	in vacuo	0.8902 0.8905	0.9916 0.9897	4893 4882 4904	5475	

Conglutin α, Yellow lupine.

at 110°	in vacuo	1.8551	2.0223	5040 5047	5528	5536
	at 110° and then in vacuo	1.8537	2.0203	5061 5067	5548	

Conglutin β, Yellow lupine.

over H_2SO_4	in vacuo	1.8530	2.0109	4873 4868 4888	5302	5376
	at 110° and then in vacuo	1.8278	2.0275	4808 4799	5341	

VICILIN. This is a globulin associated with legumin in the seeds of lentil (*Ervum lens*), horse bean (*Vicia faba*) and pea (*Pisium Sativum*). Its composition is

C, 52.29; H, 7.03; N, 17.11; S, 0.17; O, 23.40 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.
at 110°	in vacuo	0.8900 gm.	0.9727 gm.	5183 5200	5683

LEGUMELIN. This is an albumin-like protein which is found in the extracts of most leguminous seeds. This preparation was from the lentil (*Ervum lens*). The composition of legumelin is

C, 53.31; H, 6.71; N, 16.08; S, 0.97; O, 22.93 per cent.

I. Previously Dried	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.
at 110°	in vacuo	0.9154	0.9901 gm.	5209	5676

GLIADIN. This is a protein soluble in 70–80 per cent alcohol, which forms nearly one-half of the protein of wheat (*Triticum vulgare*) and rye kernels (*Secale cereale*). The composition of gliadin is

C, 52.72; H, 6.86; N, 17.66; S, 1.03; O, 21.73 per cent.

Gliadin from wheat.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.
I. over H_2SO_4	in vacuo	1.7786 gm. 1.7787 "	1.9596 gm. 1.9557 "	5229 5226 5242	5784
II. over H_2SO_4	in vacuo	1.8045 " 0.9023 "	1.9903 " 0.9928 "	5187 5181	5713
Gliadin from rye.					
over H_2SO_4	in vacuo	1.8095 " 1.8090 "	1.9925 " 1.9955 "	5184 5203 5177 5184	5717

GLUTENIN. This protein (Gluten-casein according to Ritthausen) forms about one-half of the protein matter of the gluten of most varieties of wheat, and is insoluble in neutral solvents. Its ultimate composition is nearly the same as that of gliadin but the structure of its molecule is very different, as shown by a comparison of the proportion of decomposition products yielded by these two proteins. The composition of glutenin is

C, 52.34; H, 6.83; N, 17.49; S, 1.08; O, 22.26 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.
at 110°	in vacuo	1.8072 gm.	1.9924 gm.	5134	5704
		1.8054 "	1.9977 "	5137	
				5111	5703
				5119	
				5116	

GLOBULIN. Wheat (*Triticum vulgare*). This globulin forms only a very small part of the total protein of the wheat kernel. It is contained chiefly, if not wholly in the embryo of the seed. Its composition is

C, 51.03; H, 6.85; N, 18.30; S, 0.69; O, 23.13 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.
over H_2SO_4	in vacuo	0.8925 gm.	0.9883 gm.	4759	5358
				4790	

HORDEIN. Barley (*Hordeum vulgare*). Hordein, which forms about one-half of the protein substance of the barley kernel, is readily soluble in 70–80 per cent alcohol. Its composition is

C, 54.29; H, 6.80; N, 17.20; S, 0.85; O, 20.86 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance.	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.	VII. Corrected for Difference in Moisture.
over H_2SO_4	in vacuo	1.8082 gm.	2.0025 gm.	5316	5902	5916
				5317		
	at 110° and then in vacuo	1.8043 "	1.9946 "	5349	5934	
				5361		

BYNIN. Barley malt. Bynin is the alcohol soluble protein of barley malt. Its composition is

C, 55.03; H, 6.67; N, 16.26; S, 0.84; O, 21.20 per cent.

I. Previously Dried.	II. Dried.	III. Weight of Dry Substance	IV. Weight of Substance when Burned.	V. Heat of Combustion, Calories per Gram.	VI. Calculated to Ash- and Water-free Basis.
at 110°	in vacuo	0.9006 gm.	0.9899 gm.	5262 5293	5807

The results of these determinations are given in the following table:

	C	H	N	S	O	Calories per gram
Amandin	51.30	6.90	18.90	0.43	22.47	5543
Corylin	50.72	6.86	19.03	0.83	22.56	5590
Excelsin	52.23	6.95	18.26	1.09	21.47	5737
Edestin	51.36	7.01	18.65	0.88	22.10	5635
Globulin (Cotton seed)	51.71	6.86	18.30	0.62	22.51	5596
Vignin	52.64	6.95	17.25	0.42	22.74	5718
Glycinin	52.01	6.89	17.47	0.71	22.92	5668
Legumin	51.72	6.95	18.04	0.39	22.90	5620
Phaseolin	52.57	6.97	15.84	0.33	24.29	5726
Conglutin (Blue lupine)	51.13	6.86	18.11	0.32	23.10	5475
Conglutin α (Yellow lupine)	51.75	6.96	17.57	0.62	23.10	5542
Conglutin β (Yellow lupine)	49.91	6.81	18.40	1.67	23.21	5359
Vicilin	52.29	7.03	17.11	0.17	23.40	5683
Legumelin	53.31	6.71	16.08	0.97	22.93	5676
Gliadin	52.72	6.86	17.66	1.03	21.73	5738
Glutenin	52.34	6.83	17.49	1.08	22.26	5704
Globulin (Wheat)	51.03	6.85	18.30	0.69	23.13	5358
Hordein	54.29	6.80	17.20	0.85	20.86	5916
Bynin	55.03	6.67	16.26	0.84	21.20	5807

In general the higher heats of combustion are found for those proteins which have a higher carbon content and similarly for those with a lower oxygen content. Many irregularities, however, appear in the preceding table, which are doubtless due to the different proportions of the various amino-acids which constitute the molecules of the different proteins. As we have but little knowledge of the relative proportions of these amino-acids in the proteins burned, we are not able yet to draw any definite conclusions in regard to these differences.

IS THE SALIVA OF THE DOG AMYLOLYTICALLY ACTIVE?

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(Received for publication, March 19, 1907.)

The view that the saliva of certain animals, particularly the carnivora, is devoid of amylolytic power, has been current since the classic investigations of Bidder and Schmidt.[1] With respect to the dog their statement is quite specific.[2] An equally positive and definite conclusion was drawn by Claude Bernard from his experimental observations.[3] Physiological literature since that

[1] Bidder und Schmidt: *Die Verdauungssäfte und der Stoffwechsel*, 1852, p. 14, flg.

[2] Wir haben daher neuerdings nicht nur die reinen Sekrete der betreffenden Drüsen aufgefangen, sondern auch das reine Secret der Mundschleimhaut von Hunden gewonnen. . . . Die so erhaltenen Secrete wurden, jedes für sich, mit Stärkekleister vermischt und einer Temperatur von 40° C. ausgesetzt. In keinem Falle war durch die Trommersche Probe vor 8 Stunden und auch dann nur eine Spur von Zucker nachzuweisen, und wir müssen hiernach auf's Nachdrücklichste wiederholen, dass Keinem der Secrete, durch deren Vermischung die Mundflüssigkeit gebildet wird, allein für sich bei der Umsetzung des Stärkemehls in Zucker irgend eine Fermentwirkung zugeschrieben werden könne. . . . (*loc. cit.*, p. 19 flg.)

[3] "La salive de chien pure et à l'état frais n'agit pas sur l'eau d'amidon; mais elle acquiert cette propriété lorsque, abandonnée à elle-même, elle vient à éprouver une certain degré d'altération. C'est ce que prouve l'expérience suivante. Exp.—Des salives fraîches de chien sous-maxillaire et sublinguale, très gluantes, ont été séparément mises en contact avec de l'eau d'empois d'amidon, et n'ont exercé aucune action pour changer cette substance en sucre. Au bout de deux jours, ces salives ayant été abandonées à elles-mêmes par un temps chaud et orageux, avaient complétement perdu leur viscosité, et alors elles agissaient très énergiquement sur l'eau d'amidon pour le transformer en sucre. De la salive parotidienne fraîche placée dans les mêmes circonstances n'eut pas d'action sur l'eau d'empois d'amidon, et acquit la propriété de la transformer lorsqu'elle eut subi un commencement d'alteration: d'ou il faut conclure qu'à l'état frais, les salives pures ne transforment pas l'eau d'empois d'amidon. (Claude Bernard: *Leçons sur les propriétés physiologiques et les altération pathologiques des liquides de l'organisme*, ii, p. 249, 1859.)

time has repeatedly reiterated these announcements,[1] so that we read in the recent lectures of Starling: "In dogs the mechanical action of saliva is its only one."[2]

The experience in our laboratory has never afforded any occasion to question the accuracy of this statement. It was with some surprise, therefore, that we learned from the paper of Neilson and Terry,[3] entitled "The Adaptation of the Salivary Secretion to Diet," that in all their experiments "the saliva of dogs was found to be active, but varying considerably in its amylolytic power, in different animals." Dogs fed upon a diet consisting principally of bread, with a small amount of meat broth and some ground meat are reported as invariably having a saliva with strong amylolytic powers. The authors claim that both the saliva and gland extracts of dogs fed on a mixed diet containing bread show a much greater amylolytic power than those of street dogs on an unknown diet. An experiment is reported in which a dog was fed on meat for fourteen days, the salivary glands on one side were taken out, and the animal subsequently put on a bread diet for fourteen days before removal of the remaining glands. No sugar was detected in any case within the first hour in the digestion trials with the extracts and starch paste; but on the basis of subsequent observations upon the digestive mixtures the conclusion is drawn that the glands adapt themselves to diet. It is further stated that "probably in all dogs there is an active ptyalin, but it is relatively inert as compared to human saliva."

The importance and interest which attaches to a demonstration of adaptation in digestive secretions need scarcely be emphasized. Earlier alleged evidences of such reactions in the animal organism have lately been subjected to severe criticism,[4] so that analogies in the case of the pancreas and intestine are, at the

[1] They are found, for example, in the text-books of Gamgee, Neumeister, Schäfer, Hammarsten and Abderhalden.

[2] Starling: *Recent Advances in the Physiology of Digestion*, p. 41, 1906. Fermi (*Arch. f. Physiol.*, Supplementband, p. 65, 1901) also failed to find ptyalin in dog's saliva.

[3] Neilson and Terry: *Amer. Journ. of Physiol.*, xv, p. 406, 1906.

[4] Cf. Plimmer: *Journ. of Physiol.*, xxxiv, p. 93, 1906; xxxv, p. 20, 1906; Bierry: *Compt. rend. de la soc. de biol.*, lviii, pp. 700, 701, 1905.

present moment, not well substantiated. Aside from the work of Neilson and Terry we recall only two other recent investigations which attribute amylolytic properties to dog's saliva. Henri and Malloizel have incidentally noted variations in supposed amylolytic activity in the submaxillary saliva of dogs when different stimuli were employed. They remark "La salive sous-maxillaire est toujours très peu active. Dans les cas de l'activité maximum nous avons trouvé au bout de 5 heures, 6 à 7 milligrammes de sucre."[1] When we add that under comparable conditions of experiment lymph formed five times as much sugar and that there was a parallelism between the mucin content of the digestive mixtures and their supposed activity, these experiments have little positive significance. Aside from this inconclusive evidence we have further noted an experiment by Hemmeter[2] in which dog saliva and trypsin solutions were subjected in different and separate portions to various temperatures, and thereafter their amylolytic and proteolytic power tested. Since the investigator was studying a quite different problem and used saliva as a type of amylolytic secretion one must assume that he regards the canine saliva to be active upon starch.[3]

We have made a series of observations upon dogs and cats in order to demonstrate, if possible, the native or acquired amylolytic properties of the saliva. *The experiments have failed to give evidence of any marked or characteristic digestive action upon*

[1] Henri and Malloizel: *Compt. rend. de la soc. de biol.*, liv, p. 331, 1902. Our attention was directed to this paper by Prof. A. J. Carlson.

[2] Hemmeter: *Journ. of the Amer. Med. Assoc.*, Dec. 9, 1905; *Berl. klin. Wochenschr.*, 1905, No. 44a (Ewald Festschrift). In the older literature positive results are recorded by Astaschewsky. Cf. *Jahresber. f. Thierchem.*, vii, 256, 1877.

[3] Professor Hemmeter has informed one of us (M.) that he has observed the inactivity of dog's saliva on boiled starch in some cases. He writes: "I noticed in one dog that when the saliva is collected by a sponge from his mouth and not by catheterization of the duct, that sometimes it is active and sometimes it is not. Even in dogs in which the saliva is obtained by catheterization of the duct and stimulation of the chorda tympani, the saliva is of varying amylolytic power. This is true not only of dogs, but also of human beings . . . some deep seated causes are underlying these discrepancies." Professor Hemmeter adds that he has not investigated the production of sugar in the digestions, but confined his attention to the solution of the starch.

starch paste.[1] Furthermore a careful study of the data submitted by Neilson and Terry renders their positive deductions somewhat less convincing.[2] The reacting solutions as a rule reduced Haines' solution only after comparatively long periods of digestion, and then only slightly. For example, one extract in which the reduction test showed "a trace of sugar on standing" after four hours' digestion with one gram of starch, was ascertained to contain 0.029 gram of sugar (Experiment D); whereas a comparable digestion (Experiment B) with a presumably more active extract is recorded as giving a "heavy reduction at once" after one and one-half hours, while the amount of sugar was estimated at only 0.054 gram from 1.3 grams of starch. In one experiment (B) upon a bread-fed dog[3] the saliva itself was distinctly active. With this exception, the amylase might be regarded as relatively inert, at any rate if we use the observations on the pancreatic amylase of the same animal or the saliva of other species as standards of comparison.

In our experiments upon dogs the saliva was obtained from cannulas in the ducts after stimulation of the chorda tympani, with frequent intervals of rest, unless otherwise stated. The animals were anæsthetized with ether or A. C. E., after administration of a small dose of morphine. The gland extracts were prepared with toluene water and filtered through absorbent cotton. A one per cent paste prepared from best grade arrowroot starch was used in the digestions, which were carried on in the presence of toluene, at 40° C. The progress of amylolysis was noted by applying the iodine test and Fehling's test. Where quantitative estimations were attempted the Allihn gravimetric method was applied, because we regard this procedure as rather more satisfactory than the volumetric processes where such

[1] I am informed by Prof. E. H. Starling that he also has examined both submaxillary and parotid saliva from the dog (after chorda stimulation or pilocarpine injection) a number of times, but has never obtained any amylolytic effect.—L. B. M.

[2] Plimmer also says: "The results obtained by these authors, however, are not very conclusive and further experiments on this point would be of great interest." (*Journ. of Physiol.*, xxxv, p. 21, 1906.)

[3] In the original paper B, p. 409, is labeled "*Meat-fed Dogs*"—evidently a typographical error. The weight of the second submaxillary gland on p. 410 is also apparently printed incorrectly.

mall quantities of reducing substances are present. The reducıg power of the solutions is expressed in terms of copper. This ras necessary because in several of the trials the quantity of ıaltose could not be calculated from Wein's tables, as the amounts f copper obtained fell below the limits of these tables.

PROTOCOLS.

I. A dog weighing 14 kilos was used. Diet unknown. The saliva was obtained from both submaxillary glands, and portions successively collected were mixed. The final records noted below were made after three to four days digestion at about 40° C.

(*a*) 3.15 p. m.	Mixed Saliva	20 cc.	In two hours a quantitative estimation indicated 0.0137 gram Cu in all. Three days later this was practically unchanged: 0.0141 gram Cu in all. After four days the iodine reaction showed a reddish tinge. A slight reduction could be observed with Fehling's solution.
	Starch Paste	20 "	
	Sterilized Water and Toluene	20 "	
		60 "	
(*b*) 4.05	Saliva	40 cc.	The unchanged starch interfered with the filtration of the trace of cuprous oxide precipitated. After four days the solution still gave the blue color with iodine solution and only a slight reduction.
	Starch	25 "	
	Water and Toluene	10 "	
		75 "	
(*c*) 4.15	Saliva	6 cc.	Four days later this gave a reddish blue iodine test and a slight reduction with Fehling's solution.
	Starch and Toluene	6 "	
		12 "	

(*d*) 4.25 A similar trial gave less evidence of digestion after four days.

(*e*) 4.30 5 cc. saliva + toluene. *No starch added.* On the following day this saliva alone yielded 0.0018 gram Cu in an Allihn estimation.

It will be noted that the digestive action, even as indicated by the reduction tests, was minimal at most. In (*a*), for example, despite the large quantity of saliva present, it did not increase, thus suggesting exterior causes for the reaction. The saliva itself, after standing, gave a visible reduction sufficient to account for part of the other reactions.

II. From a dog weighing 15 kilos, saliva was collected alternately from the right and left submaxillary gland, with varying strengths of stimuli (coil distance) and intervals of rest, and it was tested in portions of 5 cc. saliva + 5 cc. starch paste + toluene. Next day the nine tubes all still showed a blue reaction with iodine. Slight reduction tests were obtained, becoming somewhat more distinct in the portions of saliva last collected (after 2 hours).

III. A bitch weighing 9.5 kilos was anæsthetized with ether and A. C. E. and saliva was collected directly by blowing chloroform or ether vapor into the mouth. Digestion trials were made: 4½ cc. saliva + 4½ cc. starch paste (1) with and (2) without addition of toluene. On the following day both tubes gave a blue reaction with iodine solution and a slight reduction. Four days later (1) was clear but still gave a blue reaction with iodine (soluble starch) as well as a marked reduction test; (2) was decomposed.

IV. For quantitative estimations submaxillary saliva was collected by chorda stimulation from the same animal, and digestion trials were arranged as follows:

(1)	(2)
18 cc. Saliva.	18 cc. Saliva.
18 " Starch Paste.	18 " Starch Paste.
18 " Water.	18 " Water.
Toluene.	No Toluene.

Two days later the mixtures still gave a blue reaction with iodine despite the very large quantities of saliva used.

Copper found: (1) 0.0480 gm.; (2) 0.0696 gm.

The toluene was omitted from one of the trials in order to exclude any possible retarding action of the antiseptic on the enzyme. The slightly increased yield of copper in (2) may equally well be ascribed to the action of microörganisms. At any rate no serious inhibition is attributable to the toluene used in our experiments.

V. The dog weighed 12 kilos. Cannulas were introduced into the ducts of both submaxillary and sublingual glands. The periods of stimulation and rest were continued over three hours; the submaxillary and parotid glands were excised, minced and

extracted with toluene water and filtered through absorbent cotton.

Successive portions of saliva, etc., were mixed, *as soon as collected*, with sterilized water, starch paste and toluene. They were examined 24 hours later.

			Iodine Test.	Reduction Test.
(1)	5 cc.	Submaxillary Saliva	blue	18 mgm. Cu
	20 "	Starch Paste		
	5 "	Water and Toluene		
(2)	10 "	Submaxillary Saliva	blue	28 mgm. Cu
	10 "	Starch Paste		
		Toluene		
(3)	5 "	*Boiled* Submaxillary Saliva	blue	none
	20 "	Starch Paste		
	5 "	Water and Toluene		
(4)	1 "	Sublingual Saliva	blue	faint
	10 "	Starch Paste		
	5 "	Water and Toluene		
(5)	10 "	Submaxillary Saliva	purple	positive
	10 "	Starch Paste		
		Toluene		
(6)	15 "	Parotid Extract	red	heavy
	20 "	Starch Paste		
		Toluene		
(7)	Ditto, with Parotid Extract *boiled*,		blue	none
(8)	35 cc.	Submaxillary Extract	colorless	heavy
	40 "	Starch Paste		
		Toluene		
(9)	Ditto with submaxillary extract *boiled*,		blue	none

(10) A further experiment was tried with 7 cc. of submaxillary saliva which was *allowed to stand two hours* under cover before being mixed with 5 cc. starch paste and toluene. This mixture gave *no color with iodine* and a *heavy reduction test* on the following day.

(11) A mixture of 4.6 cc. submaxillary saliva + 10 cc. starch paste + toluene was frequently tested. After 1½ hour it began to give a faint reduction test which did not increase in the next 20 hours.

These trials indicate the nearest approach to active digestion which we have recorded.

VI. *Adaptation Experiment.* A dog weighing 14.5 kilos was kept upon a diet of bread, with a little milk and without any meat, during 4 to 6 weeks. Successive portions of submaxillary saliva were examined alone, or with equal quantities of starch paste, with and without toluene.

	(1) Saliva 20 cc. Starch Paste. Toluene.	(2) Saliva 20 cc. Starch Paste. No Toluene.	(3) Saliva 10 cc. Toluene.	(4) Saliva 20 cc. Starch Paste Toluene.
Reduction test, next day..	.0117 gm. Cu	faint	0.001 gm. Cu	faint
Iodine test, after 2 days		blue		blue
" " " 9 "		decomposed		blue
Reduction test, after 9 days		none		positive

There is no evidence here, in our opinion, that any adaptation has taken place.

VII. *Adaptation Experiment.* A bitch weighing 15 kilos had for months been kept upon a diet of meat, *cracker meal* and lard, which was regarded as very favorable for the development of an adaptation of the secretion. Saliva was collected from both submaxillary glands with varying rates of flow and intensity of stimulation (coil distance). The different portions, usually about 5 cc., were mixed with equal volumes of starch paste + toluene. After one day at 37° C. all still gave a blue iodine test together with reduction tests on standing, which we can best describe as varying from slight to distinct, without any apparent sequence. Thus the most distinct reduction test was given by a specimen obtained from the left gland with weak stimulation, while three specimens secreted under comparable conditions from the right gland showed almost no cuprous deposit.

VIII. The submaxillary saliva of a large dog was collected directly from a duct by chorda stimulation. To 10 cc. portions of starch paste an equal volume of (1) boiled and (2) unboiled saliva was added in two flasks, with toluene. Next day both mixtures still gave a blue color with iodine solution, 5 cc. gave a slight reduction in each with Fehling's solution. The deposited cuprous oxide was, if anything, larger in quantity in (2).

IX. *Experiments with Cats.* The saliva of the cat is generally regarded as devoid of amylolytic properties.[1] We have collected mixed saliva from this animal directly from the mouth by the use of ether vapor stimulation. It fails to clear starch paste or give evidence of amylolytic power. The possible influence of diet has not been considered.

We agree with Plimmer in assuming that adaptation is perhaps more readily established in connection with functions present or dormant than in the case of activities of which there are no direct indications in the species.

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[1] Cf. Cannon and Day: *Amer. Journ. of Physiol.*, ix, p. 396, 1903. Other references of interest in this connection are given in the introductory part of their paper.

The extracts from our protocols have been recorded here in somewhat greater detail than they might otherwise deserve, in order to enable the impartial observer to draw his own conclusions. Our interpretation of the observations has already been indicated. The almost uniform failure of dog's saliva, collected under variable conditions from the mouth cavity or directly from the ducts, to convert starch paste completely to or beyond the dextrin stage under the most favorable conditions of digestion seems to speak against any distinctive amylolytic activity.[1] The more nearly positive results indicated in Experiment V could not be obtained again. The experiments with the gland extracts are not so significant when we recall the wide distribution of amylases in small amounts throughout many tissues and in the blood and lymph.[2] Furthermore the demonstration of a *slight* reducing power must be interpreted with considerable caution. The saliva itself was sometimes slightly effective in this direction; and the presence of sugar in the digestions is an uncertain inference when the quantitative data approach the lower limits of accuracy. This is emphasized by the failure of the slight reducing power at times observed to increase adequately with the period of digestion. While these experiments give no evidence of a specific adaptation of the salivary glands effected by diet, the possibility of a more successful result under still other conditions cannot be denied.

[1] If one accepts the idea of a specificity of amylases as advocated by Ascoli and Bonfanti (*Zeitschr. f. physiol. Chem.*, xliii, p. 156, 1904), it might be objected that the test-starch selected was unsatisfactory. It is, of course, also possible, though unlikely, that the canine saliva contains enzymes which act upon *soluble* starch and dextrins, without converting starch paste.

[2] Cf. for example, Bainbridge and Beddard: *Bio-chemical Journal*, ii, p. 91, 1907.

A NOTE ON THE BEHAVIOR OF URIC ACID TOWARD ANIMAL EXTRACTS AND ALKALIES.

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Various animal tissues possess the power of destroying uric acid. Wiener,[1] Schittenhelm,[2] Burian[3] and others[4] have shown that this capacity varies in different species and in different organs of the same species. In a recent paper Austin[5] gives the results of an examination of ox spleen and kidney for a uricolytic enzyme. He concludes: "We have some evidence that there is a uric acid splitting enzyme in both the spleen and kidney of the ox; still the results obtained are so closely simulated by the alkali used for solution of the uric acid that the proof is not positive."

Austin's method of investigation consists in extracting the organs with water, filtering, precipitating the filtrate with twice its volume of saturated ammonium sulphate solution, removing the precipitate so formed, then dissolving it in water and dialyzing. The resulting enzyme solution was divided into 400-cc. portions and digested with 0.5 gram of uric acid dissolved in sodium hydroxide. An equal volume of a solution of uric acid in the same strength of alkali was used as a control. After three days the uric acid was found to have diminished not only in the digestions but to a nearly equal degree in the control uric acid solution. No controls with the boiled extracts were reported in any of the protocols.

[1] Wiener: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 375, 1899.

[2] Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlv., pp. 121, 161, 1905.

[3] Burian: *Ibid.*, xliii, p. 532, 1904.

[4] Ascoli: *Arch. f. d. ges. Physiol.*, lxxii, p. 340, 1898. Bendix and Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlii, p. 461, 1904.

[5] Austin: *Journ. of Med. Research*, xv, p. 309, 1906. Since the above was printed Austin has published an additional paper on this topic: *Ibid.*, xvi, p. 71, 1907.

In view of these results Austin raises the question: How much of the action of the various purin enzymes, adenase, guanase and the uricolytic enzyme, is due to the alkali used as a solvent? Such a criticism must certainly be considered if an excess of alkali is used. It is true that uric acid dissolved in even the smallest possible amount of sodium hydroxide suffers decomposition under the conditions of these digestions.[1] A different condition exists, however, in the presence of proteid. Austin suggests that in an albuminous solution, the formation of an alkali-proteid combination may protect the uric acid, although he recorded no experiments to illustrate this point.

The following simple experiment of the writer speaks for itself:

(*a*) 0.15 gram uric acid suspended in warm water was dissolved by adding 4 cc. of 2 per cent sodium hydroxide solution and made up to 400 cc.

(*b*) 0.15 gram uric acid was similarly dissolved, the diluted, filtered white of one egg was added, and the solution made up to 400 cc.

(*c*) 0.15 gram uric acid was dissolved in 40 cc. of 2 per cent sodium hydroxide solution and made up to 400 cc.

To all the solutions considerable amounts of toluene were added and all were digested at 40° C. for three days. Air was drawn through during 30 hours of that time and toluene was renewed in each digestion daily. (*a*) and (*c*) were then treated with 10 cc. of 10 per cent hydrochloric acid and concentrated to 15 cc. (*b*) was heated to boiling, coagulation effected by adding dilute acetic acid, the fluid filtered, treated with 10 cc. of 10 per cent hydrochloric acid and concentrated to 15 cc. The uric acid was then allowed to separate by crystallization and filtered on a Gooch crucible. It was washed with alcohol and ether and dried at 100° C.

Making correction for the solubility of uric acid the amounts recovered were: (*a*) 0.1085 gram; (*b*) 0.1405 gram; (*c*) no crystals of uric acid. Considering the difficulty of removing uric acid from a proteid coagulum (*b*) may be regarded as a practically quantitative recovery. The separated crystals were submitted to Kjeldahl nitrogen estimation.

0.1200 gram of the material required 13.4 cc. of acid (1 cc. = 0.00296 gram N).

Found: N = 33.03 per cent; calculated: N = 33.33 per cent.

The solutions were quite clear at the end of the digestion, showing that the uric acid had remained in solution. Any acidity which might be assumed to develop in an autolysis of animal organs and protect the uric acid, could not be present here; for

[1] Cf. Sundwik: *Zeitschr. f. physiol. Chem.*, xx, p. 335, 1894.

the alkaline eggwhite does not readily autolyze. The experiment illustrates the destructive action of excess of alkali, and the protective influence exerted by proteids.

Experimental studies which the writer has been conducting for some time with Professor Mendel[1] on the uricolytic power and purin enzymes of embryonic tissues has made it necessary to consider carefully the adverse criticism of Austin. In the light of our experience, however, his conclusions seem unjustified. For example, we have found: (1) that uric acid is not destroyed by extracts of *embryo* pig's livers; (2) that, under comparable conditions, uric acid is destroyed by extracts of *adult* pig's liver, and (3) that uric acid is not destroyed by a *boiled* extract of adult pig's liver.

METHOD: Livers obtained fresh from the slaughter house were macerated and extracted with five times their weight of toluene water during 24 hours with frequent stirring. To 500 cc. of the filtered extract, 0.15 gram of uric acid was added after being dissolved in the amount of sodium hydroxide necessary to form disodium urate. The solutions were then digested at 38° C., air being drawn through for periods varying in different experiments. The digestion mixtures were precipitated by heat with addition of acetic acid and the coagulum was redissolved in dilute alkali and reprecipitated with acetic acid. The filtrates were united and precipitated by the Krüger-Schmid[2] method with copper sulphate and sodium bisulphite. The precipitate of purin bodies so formed was decomposed with sodium sulphide, acidified with acetic acid, and the filtrate from the copper sulphide was concentrated to 20 cc. after adding 10 cc. of 10 per cent hydrochloric acid. If the uric acid did not crystallize in a pure condition, without color and admixture of foreign materials, it was redissolved in concentrated sulphuric acid, according to Horbaczewski,[3] and recrystallized by pouring into alcohol. The uric acid was weighed in a Gooch crucible after washing and drying at 100° C.

Some idea of the relative age of the embryos was obtained by measuring the length of the body. Only the largest embryos

[1] With the aid of a grant from the Carnegie Institution of Washington.

[2] Hoppe-Seyler-Thierfelder: *Handbuch der chem. Analyse*, p. 435.

[3] Horbaczewski: *Zeitschr. f. physiol. Chem.*, xviii, p. 341, 1893.

available in sufficient quantity for experiments were used. Their length varied from six to eight inches, corresponding approximately to a fœtal age of eleven to thirteen weeks.

Experiment 1. Eight-inch embryo livers extracted as above. 500 cc. digested three days with 0.15 gram uric acid dissolved in the smallest possible amount of sodium hydroxide. *0.139 gram uric acid recovered.*

Experiment 2. Seven-inch embryo livers similarly treated. *0.137 gram uric acid recovered.*

Experiment 3. Seven-inch embryo livers similarly treated. *0.122 gram uric acid recovered.*

Experiment 4. Seven-inch embryo livers similarly digested four days. *0.127 gram uric acid recovered.* This material was not collected on a Gooch crucible, but weighed in a drying bottle and subjected to Kjeldahl nitrogen estimation.

0.0820 gram of substance used 9.15 cc. acid (1 cc. = 0.00296 gram N). Found: N = 33.02 per cent; calculated: N = 33.33 per cent.

This shows that the uric acid recovered was pure. In all cases the murexide test was positive.

In contrast with this recovery of uric acid in embryo liver digestions are the following experiments with adult pig's liver.

Experiment 5. (*a*) 500 cc. extract of adult pig's liver prepared as above plus 0.15 gram uric acid digested one day. *0.048 gram uric acid recovered.*

(*b*) 500 cc. of same extract similarly digested 3 days. *0.021 gram uric acid recovered.*

(*c*) 500 cc. same extract, boiled, plus 0.15 gram uric acid digested three days. *0.139 gram uric acid recovered.*

Experiment 6. (*a*) and (*b*) prepared as (*a*) and (*b*) of preceding experiment and digested four days. No trace of a substance giving the murexide test was obtained.

Austin demonstrated as much uricolytic activity in ox spleen as in ox kidney; while Schittenhelm[1] was unable to detect such power in ox spleen. This discrepancy may be due to differences in the alkalinity of the digestions. An examination of Austin's protocols shows that in every case he used more alkali than was required to dissolve the uric acid. This excess of alkali would find in his purer enzyme solution less proteid to combine with than in a simple extract of tissues. Again, in a long process of dialysis and digestion there is considerable opportunity for bacterial contamination. In this connection, it may be noted that

[1] Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlv, p. 121, 1905.

in one of Austin's experiments, tyrosin, regarded as a product of incipient putrefaction, could be detected.

It must be admitted that the methods of testing for an uricolytic enzyme, involving as they do somewhat variable factors of alkalinity and possibility of bacterial action during a prolonged digestion, are not ideal. But experiments such as those planned by Austin in solutions poor in proteid and rich in alkali need not necessarily call into question the results obtained by the current methods. For although uric acid is destroyed by adult pig's liver, it is not changed by extract of embryo pig's livers or by a boiled extract of adult pig's liver under precisely comparable conditions of alkalinity, antisepsis, etc.

It seems more reasonable to attribute such specific differences to the variations in the enzyme content of the organs examined than to external factors.

MANGANESE, A NORMAL ELEMENT IN THE TISSUES OF THE FRESH WATER CLAMS, UNIO AND ANODONTA.

By HAROLD C. BRADLEY.

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Manganese in the tissues of normal animals may be regarded as a rare element. In traces it is known to be present in a number of marine animals, as was shown by Pichard.[1] Hoppe-Seyler[2] is authority for the statement that it is not infrequently present in human blood and in the liver: but it is neither constant in its occurrence nor significant in amount. Among the lower animals there is at least one instance on record of the normal and physiological presence of manganese in the blood and tissues. In the blood of the mollusk, *Pinna squamosa*, manganese is believed to serve a respiratory function. According to the analyses made by Griffiths,[3] manganese is present in the blood to the extent of 0.35 per cent. So unique was this discovery, however, that it has received little more than the passing interest given a curious fact devoid of any real physiological interest. We have to report in this preliminary notice what seems to be another normal example of its use in the metabolism of the fresh water clams, *Unio* and *Anodonta*, so abundant in the lakes and rivers of the Mississippi basin. While our specimens were obtained from the Madison lakes of Wisconsin only, they are thoroughly representative of the two species in every other respect, so that we are confident that specimens from other localities will exhibit this same metabolic idiosyncrasy. This point, however, will be definitely settled as soon as more material can be obtained.

While the qualitative recognition of manganese in tissues containing such considerable amounts as are present in this animal

[1] P. Pichard: *Compt. rend. de l'Acad. des. sci.*, cxxvi, pp. 550 and 1882, 1898.

[2] Hoppe-Seyler-Thierfelder: *Handbuch der chem. Analyse*, p. 39.

[3] Griffiths: *Compt. rend. de l'Acad. des sci.*, cxiv, p. 840, 1892.

is not difficult or uncertain, we have found the standard quantitative methods peculiarly inappropriate for the conditions of this research. The large amounts of calcium, magnesium, and phosphorus in the ash render the gravimetric separations tedious and uncertain. Among the volumetric methods, that of Volhard is easily the best, though as ordinarily carried out that too is unnecessarily slow. We have, therefore, modified the Volhard process in such a way that while the accuracy of the determinations is not seriously affected—if it is lessened at all—the time consumed in carrying them out is materially shortened. The details of the process are given in the hope that they may be tried out or criticised by chemists more familiar with the practical estimation of manganese, and also that biologists and biological chemists may be led to take up this problem in other localities.

The tissue is first reduced to a carbon-free ash of stable weight and composition. It has been found that the ashing of considerable amounts of the dry tissue in ordinary crucibles consumes a great deal of time; the carbon tends to assume the graphitic form and as such is but slowly oxidized, especially in the center of the ignition mass. We have, therefore, substituted the common clay pipe for the crucible, with excellent results. The bowl is filled with the ground tissue and heated over the blast lamp. The stem is connected by a T tube to the air blast, and a stream of air thus led through the ignition mass from the bottom. The amount of air may be so regulated by a stopcock that a rapid and intense oxidation goes on in the ash, accomplishing in a few minutes what requires a long period of time in a crucible under the most favorable conditions. The ash now consists chiefly of oxides, phosphates, carbonates, and sulphates of calcium, magnesium, manganese, sodium and potassium. It is moistened with nitric acid and ignited in a crucible—thus removing the carbon dioxide and leaving an ash of constant weight and fairly uniform composition.

The ash, pulverized and sampled, is weighed out for analysis. From 1.0 to 0.5 gm. are taken as a rule. This is dissolved in 5 cc. of concentrated hydrochloric acid to which about 1 cc. of nitric acid is added. The mixture is boiled till the bulk of free chlorine is removed and the solution is a clear pale yellow. If the ashing process has been complete no insoluble residues will remain. The solution is then transferred quantitatively to a graduated flask and diluted to the mark. A convenient dilution is found to be 250 cc., though a 100 cc. flask or a 500 cc. flask may be used equally well. Aliquots are then taken and transferred to Erlenmeyer flasks or covered beakers and an excess of pulverized zinc carbonate is added. The mixture is quickly brought to a boil, the spatters on the cover washed back into the beaker, and the manganese precipitated as

manganese dioxide by titrating with $\frac{N}{10}$ potassium permanganate till a permanent pink indicates the completion of the reaction. In practice the potassium permanganate is run into the first aliquot a few cubic centimeters at a time with stirring, until the reaction is nearly complete—and this can be judged quite accurately by the color of the solution. The contents of the beaker are then brought to a boil again. The hydrated manganese dioxide separates out at once as a brown precipitate and carries down with it the suspended zinc carbonate, leaving a clear supernatant liquid. The final additions of potassium permanganate are made drop by drop till the end point is reached. The second aliquot is added to the first with more zinc carbonate, brought to a boil and titrated rapidly as before. If the first two aliquots do not agree to 0.2 cc. a third is taken. Finally the total potassium permanganate used for the sum of the aliquots is made the basis for calculating the amount of manganese present in the sample of ash.

If a $\frac{N}{10}$ solution of potassium permanganate is used the calculation of results is very readily made. From the reaction of potassium permanganate in neutral solutions, it is evident that 1 cc. of the standard solution is equivalent to 0.00165 gm. of manganese.

Examination of the Method. The points of departure from the original Volhard process are in omitting the concentration of the solution with sulphuric acid, in substituting zinc carbonate for zinc oxide and in omitting to filter off the suspended zinc carbonate or zinc oxide before performing the titration. Further, the acid is neutralized only in the aliquots taken instead of in the entire solution. Since the ash is readily soluble in hydrochloric acid or aqua regia, there seems no advantage to be gained from the evaporation with sulphuric acid and its omission saves time. The substitution of zinc carbonate for zinc oxide has an advantage in that the degree of acidity can be judged at once by the effervescence, while this can be told much less readily when the oxide is used. If ordinary precautions are taken during neutralization and the subsequent heating, no loss by spattering need be experienced; the neutralization is rapid, complete, and the excess of the carbonate does not in any way affect the results, so far as we have been able to observe. The omission of the filtration is obviously advantageous if the end point can be determined accurately in the presence of the suspended zinc carbonate. Our experience leads us to believe that the end point is quite as sharp with the zinc salt there as when it is absent, while its presence insures the complete neutrality of the solution—a most important point in this reaction.

Finally, however, we must judge of a process by its actual performance under varying conditions, and while we have not subjected this to any exhaustive series of tests, the following table will indicate its general reliability under the conditions likely to be met in this work. No attempt has been made to carry out the tests of the method more accurately or carefully than the subsequent determinations on ash samples, since we wish to know not so much the accuracy of the method when employed

with extraordinary care and pains, but its limitations and the magnitude of its errors when applied to every-day analyses, with every-day speed and care. The figures, therefore, indicate its reliability when pushed to the full speed of routine determinations.

The samples of manganese were taken by measuring out definite portions of a standard potassium permanganate solution. This was decomposed by boiling with hydrochloric acid till pale yellow. The solutions were then made up to known volumes and analyzed as described before.

The effects of various amounts of zinc carbonate were tried, and the effect of varying the dilution. The amount of hydrochloric acid was kept fairly constant. A blank determination was first carried out to determine whether the zinc carbonate and other reagents used were free from reducing compounds. A single drop of the potassium permanganate solution sufficed to give a permanent pink to the blank titration.

The results are all low and while the errors are considerable they are all of the same order of magnitude and in the same direction. There is no appreciable effect of varying the dilution or the amount of zinc carbonate. The ultimate error is on the average no greater than the normal variations in samples. We may conclude, therefore, that the method as outlined is reliable enough for the purposes of proximate analysis. The figures in Table II will be uniformly low, but where from 1.0 to 0.5 gm. of ash are taken, the error will approximate 0.2 per cent of the total manganese—an amount of no significance where the normal individual variations are so large among the specimens.

Having established the efficiency of the method for our analyses, a series of determinations was made on the samples obtained. To establish the normality of the manganese in these species of mollusks, the following questions must be definitely answered: 1. Is manganese always present in the animal's tissues? 2. Is it present in significant amounts and subject to variation within physiological limits? 3. Is it a characteristic that is transmitted from generation to generation—that is, do the eggs or young exhibit the same idiosyncrasy? These questions must be solved as a preliminary to the further investigation of the physiological functions of the element in the metabolism of these mollusks.

The specimens were obtained from different localities of the Madison lakes. The entire tissues of from 12 to 24 specimens were dried and ground up for each of the samples I, II and III. The ovaries of the females were found to be full of eggs and these could readily be separated from the other tissues. In the other samples, therefore, the eggs were removed and analyzed sepa-

TABLE I.

No.	$\frac{N}{10}$ $KMnO_4$ taken.	Manganese taken.	Dilution.	Aliquots.	$ZnCO_3$ Added.	Titration. $\frac{N}{10}$ $KMnO_4$	$KMnO_4$ Calculated for Total.	Manganese Found.	Error in gms.	Percentage Error. $\frac{\text{Error} \times 100}{\text{Gms. Mn taken.}}$
	cc.	*gms.*	*cc.*	*cc.*	*gms.*	*cc.*	*cc.*	*gms.*		
1	25	.0275	100	25	1	4.15	16.33	.02695	−.00055	2.0
				50	2	8.10				
2	25	.0275	100	25	2	4.05	16.20	.02673	−.00077	2.8
				50	4	8 10				
3	25	.0275	100	25	1	4.20	16.26	.02684	−.00066	2.4
				50	2	8.00				
4	25	.0275	100	25	2	4.10	16.40	.02706	−.00044	1.6
				50	4	8.20				
5	25	.0275	250	100	4	6.55	16.25	02681	−.00069	2.6
				100	4	6.65				
6	25	.0275	500	200	2	6.60	16.56	.02733	−.00017	0.6
				200	2	6.65				
7	50	.0550	500	200	2	13.20	33.00	.05445	−.00055	1.0
				200	4	13.20				

rately, the remaining tissues dried, mixed and pulverized as before. Each of the samples IV and V represent the average composition of the tissues of some two dozen clams, minus the eggs of the females present. Samples III, IV and V were subjected to a starvation period of two, four and six weeks, respectively. This should insure the elimination of any food residues containing manganese from the alimentary tract, which might otherwise introduce a doubt as to the real significance of the metal in I and II. Separate determinations of the ash, content of each sample were made in platinum, igniting to constant weight after moistening with nitric acid. The results are summarized in the table below.

TABLE II.

Sample.	Per cent of Ash in Tissue.	Per Cent of Mn in Ash.	Per Cent of Mn in Tissues.	Remarks.
I	21.63	4.20 4.20	0.931	freshly collected
II	26.00	4.55 4.60	1.19	freshly collected
III	21.94	4.35 4.24 4.11 4.03	.929	starved two weeks
IV	17.88	5.76 5.64 5.82	1.02	starved four weeks
V	13.88	4.24 4.42	.601	starved six weeks
eggs IV	39.55	1.49 1.69	.633	starved four weeks
V	35.20	2.32	.818	starved six weeks

Decided variations in the ash content are found. In the first three samples this is not significant since the eggs with their normally high percentage of inorganic salts were not separated. Samples IV and V, however, seem to indicate the steady elimination of inorganic material during starvation. Up to the last sample when the animals were reduced to a pathological condition by long starvation and the presence in the vessels contain-

ing them of their excretory products, no decided fluctuation of the manganese was observed.

We may conclude, therefore, that so far as the specimens from the Madison lakes are concerned, manganese is a normal constituent of the tissues; it has always been present in the specimens examined, it is present in considerable quantities and varies within comparatively narrow limits—well within the limits of variation of the total inorganic constituents of the tissues. It is also present in the eggs. Further extensions of this investigation are contemplated when material is available, with a view to determining variations produced by local environment, the sources of the metal in the food of the clams, and the physiological functions of the compounds containing the manganese in the tissues.

THE QUANTITATIVE ESTIMATION OF EXTRACTIVE AND PROTEIN PHOSPHORUS.[1]

By W. KOCH.

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(Received for publication, January 16, 1907.)

The different combinations of phosphorus to be found in a given tissue may be divided into three main groups:

1. *Protein phosphorus* or phosphorus in combination with protein, including nucleoprotein and phospho-proteins or nucleo-albumins, insoluble in water especially after treatment with alcohol;

2. *Lecithin* and *kephalin phosphorus* or phosphorus in combination with fat and a nitrogen complex, soluble in alcohol and ether, but insoluble in acid chloroform water;

3. *Extractive phosphorus*, including inorganic phosphates and the simpler combinations of phosphoric acid, such as glycero-phosphoric acid, phytin or diethoxy-diphosphoric acid, and a number of related compounds as yet little investigated, all of which are soluble in water and partly soluble in dilute alcohol.

In a previous paper[2] a method for the estimation of lecithin and kephalin phosphorus was described. In the following pages are given methods for the determination of extractive and nuclein phosphorus, which can be carried on at the same time and with the same material as the lecithin estimation.

EXTRACTIVE PHOSPHORUS.

A considerable portion of this form is found in the filtrate from the lipoids precipitated with acid chloroform as described in the above mentioned publication. Whether any of this phosphorus

[1] These methods were used in the investigation with H. S. Reed, published in vol. iii, p. 49, of this *Journal*.

[2] Koch and Woods: This *Journal*, i, p. 203, 1905.

is inorganic cannot be determined. Schulze[1] in several of his publications emphasized the fact that absolute alcohol and ether do not dissolve inorganic phosphates. He is dealing, however, with relatively dry plant tissues and not with moist animal tissues which necessarily dilute the alcohol. The separate estimation of inorganic phosphates has not been attempted in these methods, as the danger of hydrolyzing simple organic combinations of phosphoric acid seemed too great to promise reliable results.

The following table gives an idea of the amount of phosphorus, not lecithin or kephalin, dissolved out by alcohol and ether from brain tissues.

TABLE I.

PHOSPHORUS IN FILTRATE FROM LIPOID PRECIPITATE. ALCOHOL-ETHER-WATER-SOLUBLE PHOSPHORUS.

Number of Case.	In Per Cent of Dry Tissue.	In Per Cent of Total Extractive P.	In Per Cent of Total P.
34♀	0.68	37.8	4.8
35♀	0.63	32.1	4.5
44♀	0.75	36.0	5.4

The remaining portion of the extractive phosphorus is to be found in the portion of the tissues insoluble in alcohol and ether and must be removed by treatment with water to which a little chloroform has been added to prevent bacterial action. Noël Paton[2] recommends dilute acid for this extraction, but does not make it clear whether he altogether avoids the possibility of breaking up more complex substances. Control experiments have shown that in the case of the brain about five or six extractions are sufficient to remove all the phosphates that can be removed. The following table gives the results.

TABLE II.

WATER-SOLUBLE PHOSPHORUS IN RESIDUE INSOLUBLE IN ALCOHOL AND ETHER. ALCOHOL-ETHER-INSOLUBLE, WATER-SOLUBLE PHOSPHORUS.

Case.	In Per cent of Dry Tissue.	In Per Cent of Total Extractive P.	In Per Cent of Total P.
34♀	1.12	62.2	7.8
35♀	1.33	67.9	9.5
44♀	1.33	64.0	9.6

[1] Schulze, E.: *Zeitschr. f. physiol. Chem.*, xx, p. 225, 1904.

[2] Noël Paton, D.: *Report of Investigations on the Life History of the Salmon in Fresh Water.* Fishery Board for Scotland, p. 143, 1898.

PROTEIN PHOSPHORUS.

The phosphorus compounds present in the tissues after extraction with alcohol, ether and water can only be nucleins, phospho-proteins and tricalcium phosphates. The latter compound is not usually supposed to be present in appreciable amount in tissues except under pathological conditions and can therefore be neglected in the case of brain tissues. If calcium is present it would be more likely to exist as a calcium protein compound. Extraction with dilute acid might be used where calcium phosphate is suspected but this procedure so swells the tissues that complete removal of the adhering liquid becomes very difficult. Besides there is the danger of rendering the alcohol-coagulated protein again soluble.

The following table gives some of the results:

TABLE III.

PHOSPHORUS IN INSOLUBLE RESIDUE. ALCOHOL-ETHER-WATER-INSOLUBLE OR PROTEIN PHOSPHORUS.

Case.	In Per cent of Dry Tissue.	In Per Cent of Total P.
34♀	0.81	5.6
35♀	0.86	6.1
44♀	0.85	6.1

A comparison of Tables I, II and III will show that about 80 per cent of the total phosphorus remains to be accounted for. This is represented by lipoid phosphorus which, in the case of corpus callosum here analyzed, is present in large amount.

DESCRIPTION OF METHOD.

About 10 grams of the moist tissue are extracted with alcohol and ether as directed in the paper on the "Estimation of the Lecithins." The *residue, insoluble in alcohol and ether*, is dried at 102° C. to constant weight, transferred to a 300 cc. Jena flask and extracted six times with about 100 cc. of water to each extraction. Every extraction should extend over 24 hours; plenty of chloroform must be added and the mixture occasionally shaken to prevent bacterial decomposition. The filtrates are evaporated in a platinum dish and dried to constant weight. The residue represents the *salts and extractives, insoluble in alcohol and ether*

and soluble in water. The dried residue is ignited in the platinum dish, surrounded by an outer larger platinum dish which is heated to bright redness, until a nearly white ash is obtained. If the inner dish does not come in direct contact with the outer dish there is no danger of volatilizing chlorids. This residue is the *alcohol-ether-insoluble, water-soluble ash*. The difference between this ash and the residue on evaporation gives the *alcohol-ether-insoluble, water-soluble, organic extractives*. The ash is moistened with 1.5 cc. of nitric acid, dissolved in water, diluted to 100 to 200 cc. and phosphorus estimation made by the molybdate method. This gives the *alcohol-ether-insoluble, water-soluble extractive phosphorus*. (Table II.)

The residue left above, insoluble in water after six extractions, is burned with nitric and sulphuric acids and the phosphorus estimated. In case calcium is present this must also be estimated in a separate sample. The phosphorus method is described in detail in a previous paper.[1] This phosphorus is called the *alcohol-ether-water-insoluble*, or *protein phosphorus*. (Table III.)

The alcohol and ether solutions obtained by the extraction of the moist tissue are treated as directed in the paper above referred to. If the emulsification and precipitation have been properly carried on, the solution in the 100 cc. graduated flask should be clear in two or three days. An excess of fat in the tissue interferes seriously with this clearing and had best be overcome by the presence of a large amount of chloroform (8 to 10 cc.) and the addition of 2 cc. instead of 1 cc. of hydrochloric acid. The amount of chloroform added must be *carefully measured and recorded*. After the solution has begun to clear and has been made up to the 100 cc. mark of the graduated flask, it is shaken and allowed to stand until the precipitate has settled. After settling the solution is filtered through a dry filter paper into a dry 100 cc. graduated cylinder. As much of the water as possible is decanted from the chloroform, but it is better not to pour any of the chloroform on the filter, as it may pass through and lipoids be thus lost. Instead of washing the chloroform containing the lipoids with acid water as previously directed, it is better to allow the filter to drain and then *read the volume of the filtrate*,

[1] Koch and Woods: *loc cit.*

which should be perfectly clear and transparent. An aliquot part of the filtrate, usually 80 cc., is evaporated in a platinum dish and dried to constant weight at 102° C. This gives the *extractives and salts soluble in alcohol, ether and water*. The residue is ignited as directed above and the ash is the *alcohol-ether-water-soluble ash* while the difference between this and the residue obtained at 102° C. represents the *alcohol-ether-water-soluble extractives*. The ash is again moistened with ½ cc. of nitric acid, diluted to 100 to 200 cc. and phosphorus estimated. This is the *alcohol-ether-water-soluble extractive phosphorus*. (Table I.)

DISCUSSION AND CALCULATION.

In order to illustrate the method of calculation it is best to take a sample analysis as follows:

RECORD OF ANALYTICAL RESULTS.

Case 34 ♀ Corpus Callosum.

Water*	70.37	per cent		Weight of sample	9.9038 grams
1. Insoluble residue†	8.68	"	"	Weight of residue insoluble in alcohol and ether	0.8886 "
				Weight of residue from six extractions	0.0289 "
2. Lecithins	4.00	"	"	Weight of residue, insoluble in alcohol, ether and water	0.8597 "
3. Kephalins	4.27	"	"	Phosphoric acid in this residue gave	0.0085 " $Mg_2P_2O_7$
4a Extractives	0.15	"	"		
5a Extractives	1.03	"	"	Residue from six water extractions	0.0289 grams
4b Ash	0.14	"	"	4b Ash on ignition	0.0142 "
5b Ash	0.45	"	"		
6 Sulphur Compound*	1.40	"	"	4a Extractives	0.0147
				Phosphoric acid gave	0.0117 " $Mg_2P_2O_7$

Protein P .....0.024 per cent.
Total Extract P...0.053 " "

To precipitate lipoids 3 cc. chloroform were used. The filtrate measured 76 cc.; 70 cc. were evaporated.

Residue	0.1060 grams
5b Ash on ignition	0.0323 "
5a Extractives	0.0737 "
Phosphoric acid in this residue gave	0.0051 " $Mg_2P_2O_7$
2 Lecithin P gave	0.0551 grams
3 Kephalin P gave	0.0697 "

* Separate estimation.
† This essentially represents the total proteids and any glycogen that may be present in the tissues.

The method of calculating the above results is as follows:

5a and 5b alcohol-ether-water-soluble extractives and ash are calculated to 97 cc. (thus correcting for the volume of the chloroform)

$$\frac{0.0737 \times 97 \times 100}{70 \times 9.9038} = 1.03; \qquad \frac{0.0323 \times 97 \times 100}{70 \times 9.9038} = 0.45$$

Alcohol-ether-water-soluble, extractive phosphorus.

$$\frac{0.0051 \times 97 \times 100 \times 62}{70 \times 9.9038 \times 222.7} = 0.020$$

The same method applies to the calculation of the alcohol-ether-water-soluble phosphorus. The filtrate obtained was only 74 cc., but as only 5 cc. of chloroform were used, the remaining watery solution must have remained clinging to the rather spongy mass of fat and chloroform. Some little solution goes to moisten the filter paper, but this partly counterbalances whatever chloroform may have gone into solution. This method of calculation is not absolutely accurate, but comes sufficiently near, considering the variations to which the material is liable in any case.

3. *The kephalin* receives a correction for the amount of phosphorus in the liquid clinging to the lipoid precipitate, in this case 95 − 74 = 21 cc., equivalent to 0.06 milligram of phosphorus or 0.08 per cent of kephalin.

Theoretically the correction should be distributed between the lecithin and kephalin, the values of both of which it affects. As it is not improbable, however, that the water-soluble phosphoric acid derivatives with which we are here dealing form insoluble lead salts in ammoniacal alcohol solution, it was deemed best to apply the whole correction to the kephalin. The kephalin in the case of brain tissues receives a further correction for the phosphorus found in the sulphur compound.

In the light of later results it was found advisable to change the method for the estimation of kephalin outlined in a previous paper as follows:

This *Journal, i, p. 208, line 7. Substitute:* allow to remain on water-bath until there is no more smell of ammonia. The *flask* is then set aside to cool, after replacing the alcohol which has evaporated.

p. 208, line 12. Read: and the precipitate washed *once* with hot alcohol.

p. 208, line 21. Experience has shown that it is preferable to burn the filter paper with the precipitate.

In the muscle tissues of animals containing a solid fat the correction for the kephalin on account of filtrate adhering to lipoid precipitate may become rather large. It can, however, be easily reduced by making the precipitate in a larger flask (200 cc. or 400 cc.) and thus diluting the adhering filtrate.

The method above outlined should be capable of more general application to normal and pathological material. Such a study on the nervous system is soon to be published. This investigation was aided by grant from the Rockefeller Institute for Medical Research.

DAY AND NIGHT URINES.

By E. OSTERBERG and C. G. L. WOLF.

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(Received for publication, March 1, 1907.)

The investigation of the excretory relationships during the hours of sleep and of waking was one of the earliest problems attempted in metabolism. Schweig,[1] in 1843, endeavored to follow out the course of uric acid during eight-hour periods, and came to the conclusion that this substance was eliminated in least quantity during the night, and most during the midday eight-hour period. Vogel[2] followed the hourly excretion, and found that less urine was passed during the night than during hours of activity. In commenting on Vogel's results Speck[3] attempted to show that the increase in the excretion during the day was due to the intake of fluids during this time. On the other hand, Böcker[4] observed a nocturnal polyuria, and asserted that the elimination by the skin was more profuse during inactivity.

Of the more recent work on this subject one has the observations of Quincke,[5] who determined that the urine was without exception more abundant during activity. In following the elimination, he found that excretion was most pronounced during the first three hours of waking. He explains his results as being due, either to decreased resorption of fluid from the intestinal tract during sleep, or to retention in the organs, or that the kidney secretion is itself diminished, either from lessened blood supply, or contraction of the vessels. His results have

[1] Schweig: Cited by Speck.
[2] Vogel: Cited by Speck.
[3] Speck: *Arch. f. exp. Path. u. Pharm.*, xv, p. 109, 1881.
[4] Cited by Speck: *Ibid.*, p. 108.
[5] Quincke: *Ibid.*, vii, p. 115, 1877.

since been confirmed by Breisacher,[1] Lähr[2] and others. It appears, however, that in certain affections such as renal and cardiac disease, one may have a nocturnal polyuria which is very distinct.[3]

As far as we are aware no complete analyses of normal urine in which the nitrogen elimination has been compared with that of sulfur are on record. In the course of an investigation of pathological urines in which it was often difficult to obtain complete samples of the twenty-four hour excretion, we thought it of interest to make complete analyses of twelve-hour periods under ordinary conditions of diet. We hoped in this way to obtain some data of comparative value which were not available. For that reason the subject was not put on an accurate standard diet. In the first series he partook mainly of cereal food, and in the second, a fairly liberal amount of lean meat was eaten. The collection of the urines was made in alternating twelve-hour periods, during one of which the subject rested in bed, and in the other performed ordinary laboratory work. It was thought advisable to collect the urines three hours after the period had been finished, in order to compensate for lag in excretion.

The recent work of Leathes on the diurnal variation in the excretion of uric acid was published some time after these experiments were performed, and as our results corroborate those obtained by him, it seemed worth while to publish the following analyses of day and night urines, although it is realized that the series is not as complete as it might be. We hope on a future occasion to go into the matter in greater detail.

The subject of the experiments was a man, forty years of age, weighing seventy kilograms. The amount of exercise consisted in a walk of about four miles each day, and eight hours laboratory work. The heaviest meal of the day was taken at 7 p. m., and the rest period was from 8 p. m. to 8 a. m. The urines were collected at 11 p. m. and 11 a. m.

The following tables give the results of the analyses of the urine.

[1] *Arch. f. Physiol.*, p. 321, 1891.

[2] *Arch. f. Psychiatrie*, lxvi, p. 286, 1899.

[3] *Deutsch. Arch. f. klin. Med.*, lxviii, p. 175, 1900; *N. Y. Med. Journ.*, February, 1904; *Münch. med. Wochenschr.*, No. 52, 1896.

TABLE I.

Periods I, III, V and VII—activity.
Periods II, IV, VI and VIII—rest.

Period.	Volume *S. G. 10—*	Total Nitrogen.	Ammonia Nitrogen *Amm. N. : T. N.*	Urea Nitrogen *Urea N. : T. N.*	Creatinin Nitrogen *C. N. : T. N.*	Creatin Nitrogen *C'. N. : T. N.*	Uric Acid Nitrogen *U. A. N. : T. N.*	Undetermined Nitrogen *Undt. N. : T. N.*
I	540	5.240	0.205	4.440	0.235	0.035	0.050	0.239
	23		*3.9*	*84.7*	*4.5*	*0.6*	*0.9*	*4.5*
II	560	5.300	0.157	4.558	0.216	0.028	0.028	0.213
	21		*2.9*	*85.9*	*4.0*	*0.5*	*0.5*	*5.9*
III	510	5.100	0.148	4.300	0.230	0.025	0.046	0.351
	22		*2.9*	*84.2*	*4.5*	*0.5*	*0.9*	*6.8*
IV	480	5.090	0.163	4.310	0.198	0.019	0.038	0.362
	24		*3.2*	*84.6*	*3.8*	*0.3*	*0.7*	*7.1*
V	680	7.344	0.245	6.283	0.272	0.034	0.075	0.435
	24		*3.3*	*85.6*	*3.7*	*0.4*	*1.0*	*5.9*
VI	500	7.605	0.285	6.605	0.235	0.025	0.070	0.385
	26		*3.7*	*86.9*	*3.0*	*0.3*	*0.9*	*5.0*
VII	550	8.366	0.551	6.996	0.246	0.028	0.039	0.506
	27		*6.6*	*83.6*	*2.9*	*0.3*	*0.4*	*6.0*
VIII	590	8.714	0.301	7.558	0.219	0.024	0.100	0.412
	26		*3.4*	*86.4*	*2.5*	*0.2*	*1.1*	*4.7*

TABLE II.

Periods I, III, V, VII—activity.
Periods II, IV, VI, VIII—rest.

Period.	Total S.	Total SO_4 *T. SO_4 : T. S.*	Akal. SO_4 *A. SO_4 : T. S.*	Ether. SO_4. *E. SO_4 : T. S.*	Neut. SO_4 *N. S. : T. S.*
I	0.535	0.418	0.376	0.042	0.117
		78.10	*70.25*	*7.85*	*21.90*
II	0.491	0.410	0.372	0.038	0.081
		83.50	*75.75*	*7.75*	*16.50*
III	0.530	0.413	0.372	0.041	0.117
		77.93	*70.91*	*7.74*	*22.07*
IV	0.491	0.394	0.354	0.040	0.097
		80.24	*72.10*	*8.14*	*19.76*
V	0.725	0.604	0.554	0.050	0.121
		83.30	*76.40*	*6.90*	*16.70*
VI	0.607	0.849	0.447	0.042	0.118
		80.55	*73.63*	*6.92*	*19.45*
VII	0.683	0.578	0.535	0.043	0.105
		84.65	*78.35*	*6.30*	*15.35*
VIII	0.599	0.503	0.457	0.046	0.096
		84.04	*76.36*	*7.68*	*15.96*

THE VOLUME. No regularity in the volume of the urine was observed. On each diet, the excesses of excretion are equally divided between the night and morning urines.

TOTAL NITROGEN. The same effect is noted as with the volume. The elimination of nitrogen does not correspond to the volume, for in one period the greater amount of nitrogen was excreted with the less volume of urine.

UREA. The urea practically follows the course of the total nitrogen. In Periods V and VI the positions are reversed, but the difference is almost inappreciable. In all cases the relation of urea to total nitrogen is higher during sleep than in activity.

AMMONIA. In all instances the ammonia is higher during sleep than during waking, except in one instance, which occurs during the low protein diet.

CREATININ. It was thought that one might obtain some information regarding the influence of muscular activity on the elimination of creatinin, which is still under discussion. The results of von Hoogenhuyze and Verploegh[1] would lead one to infer that this substance is not increased by muscular activity. On the other hand, the recent estimations of Leathes[2] point to an increase. Our own results point to an increase during the day. The amount is always higher during hours of work. The small number of observations here recorded are not sufficient to do more than show that the question is not at all settled. One difference between our experiments and those of the Dutch observers may be noted. The latter made experiments which showed the difference between severe muscular work and ordinary activity. Our experiments are those of normal activity and complete rest.

CREATIN. It will be observed that throughout this series a small amount of creatin is present. This substance is not supposed to be a constant constituent of normal urine, yet the subject of these experiments was as far as we are aware in perfect health. The only anomaly in his metabolism was shown some time previously in experiments in the respiration calorimeter in a tendency to rapid elimination of nitrogen and what appeared

[1] von Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, xlvi, p. 415, 1905.

[2] Leathes: *Journ. of Physiol.*, xxxv, p. 125, 1906.

to be mental excitement.[1] In each set the elimination of creatin was greater during the day than at night.

URIC ACID. The uric acid appears to follow to some extent the course of the creatinin, although during one period, the elimination is lower during a waking period. From what is known regarding the excretion of this substance after the intake of purin-containing food, one can scarcely expect to find any dependable regularity in experiments of such short duration as these,[2] particularly when it is remembered that in the second set a considerable quantity of meat was eaten.

UNDETERMINED NITROGEN. This fraction of the excretion is eliminated in greater quantity during work, with the exception of one of the periods, where the excess was not very great.

SULFUR. The elimination of sulfur presents more regularity than that of the nitrogen. The excretion of total sulfur, total sulfates, and neutral sulfur is uniformly higher during waking than during sleep. The ethereal sulfur is the exception. During low protein diet the elimination was higher. During high protein diet the elimination was lower in the periods in which work was done. The difference is, however, not considerable.

[1] Atwater and Benedict: *Transactions of the National Academy of Sciences*, viii, p. 426.

[2] Soetbeer: *Zeitschr. f. physiol. Chem.*, xl, p. 25, 1903.

AMMONIA IN MILK AND ITS DEVELOPMENT DURING PROTEOLYSIS UNDER THE INFLUENCE OF STRONG ANTISEPTICS.

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(*Contributions from the Havemeyer Laboratories, Columbia University, No. 136.*)

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Both Raudnitz[1] and Stohmann[2] quote Latschenberger[3] to the effect that cow's milk contains 0.02 per cent of ammonia. The apparent acceptance of this estimate by such authorities, and the fact that it does not seem to have been corrected in biochemical literature, leads us to record here some of the data obtained in the course of an investigation, the ultimate object of which is to throw light upon the nature of the changes occurring in milk when subjected to different methods of preservation.

Ammonia in Normal Cow's Milk.

For the determination of ammonia in milk we have used a slight modification of the Boussingault-Schaffer method.[4] A description of the method and of the experiments which led us to adopt it for this work has already been published.[5] In outline the process consists in adding to the milk an equal volume of methyl alcohol and a small amount of sodium carbonate and distilling the ammonia at 60° to 65° under reduced pressure into receivers containing an excess of standard acid. No difficulty is found in completely removing the preformed ammonia by

[1] Bestandteile, Eigenschaften und Veränderungen der Milch, *Ergebnisse der Physiologie*, 2, i.

[2] *Milch- und Molkerei-produkte*, Braunschweig, 1898.

[3] *Monatsh. f. Chem.*, v, p. 129; *Jahresber. f. Tierchem.*, p. 222, 1884.

[4] *Amer. Journ. of Physiol.*, viii, No. 4, 1903.

[5] *Journ. Amer. Chem. Soc.*, xxvii, p. 124, 1905.

this method; the only error which is likely to occur is that a small amount of ammonia may be produced by cleavage of organic compounds during the distillation. To prevent this the mixture of milk and methyl alcohol may be saturated with salt before heating. Fresh milk yields so little ammonia both when distilled with and without salt that the observed differences may be largely accidental. Stale milk, however, may show a considerable difference, indicating the presence of notable amounts of proteolytic products in which the amino-groups are less firmly bound than in the original proteid.

The average percentages of ammonia yielded by commercially fresh milk from different sources when distilled with and without salt are shown in the following table:

Source.	Number of Samples.	Ammonia. Without Salt. Per Cent.	Ammonia. With Salt. Per Cent.
High class dairy producing certified milk exclusively	2	0.0003	0.00015
Dealer B	1	0.0005	0.0003
Dealer M	1	0.0004	0.0002
Dealer T	5*	0.0005	0.0002*
Dealer W	10	0.0008	0.0004
Dealer E	6	0.0008	0.0005
Grocery	3	0.0008	0.00045
Average	28	0.0007	0.0004

* Only two of these five samples were distilled with salt.

The milk obtained from grocery was dipped from a large can, all of the other samples were bottled milk.

Since each of the 28 samples examined probably represented the mixed product of many cows it is reasonably safe to accept these figures as showing the ammonia content of cow's milk in general as sold in New York. Such milk evidently averages less than 0.001 per cent of ammonia and probably less than 0.0005 per cent. Perfectly fresh and clean milk doubtless contains considerably less than commercial milk. We have rarely found over 0.001 per cent and never over 0.002 per cent of ammonia in market milk.

Formation of Ammonia During Proteolysis.

When milk is kept at laboratory temperature so that the bacteria which it contains are allowed to develop unchecked there is usually an increase both of (preformed) ammonia and of those

proteolytic products which yield ammonia by cleavage under the conditions of distillation described above, so that the percentages of ammonia yielded with and without salt are larger and the difference between these percentages is also usually larger than in the fresh sample. Thus a sample of milk analyzed as soon as purchased and again five days later gave the following results:

	Ammonia Found by Distillation.		
	With Salt. (Preformed Ammonia.) Per Cent.	Without Salt. Per Cent.	Difference. ("Cleavage" Ammonia.) Per Cent.
Milk as purchased.....	0.0005	0.0008	0.0003
Same 5 days later.....	0.0027	0.0036	0.0009

The addition of an efficient antiseptic retards but does not stop proteolysis. Examination of duplicate portions of the same milk kept with and without chloroform yielded the following results:

	Ammonia Found by Distillation.		
	With Salt. (Preformed Ammonia.) Per Cent.	Without Salt. Per Cent.	Difference. ("Cleavage" Ammonia.) Per Cent.
Portion with no preservative (examined when 5 months old)	0.0185	0.0267	0.0082
Portion with 3 per cent chloroform (examined when 1 month old)	0.0051	0.0043	0.0008

Here the suppression of bacterial action by means of chloroform diminished the formation of those proteolytic products which yield the "cleavage" ammonia, much more than it diminished the formation of ammonia itself. This is confirmed by the fact that other samples preserved with chloroform tend to show a lower proportion of "cleavage" to preformed ammonia than in the average of samples kept without preservative.

A similar effect was found by comparison of two samples from the same source, one of which had received a sufficient, the other an insufficient, amount of formaldehyde. When examined the samples were three to four years old and in each case very extensive proteolysis had occurred,[1] over 60 per cent of the proteids having been digested to products not precipitable by tannin.

In one sample the action of bacteria appeared to have been

[1] The nature of the proteolysis which takes place under such conditions has been discussed in a previous paper. *Journ. Amer. Chem. Soc.*, xxviii, p. 189, 1906.

quite thoroughly inhibited, as the percentage of lactose had not decreased and scarcely any odor had developed. The other sample had a very pronounced odor resembling that of strong cheese and was found to contain less than half the original percentage of lactose.

On distillation for ammonia with and without the addition of salt the following results were obtained:

	Preformed Ammonia. Per Cent.	"Cleavage" Ammonia. Per Cent.	Total Per Cent.
Sample containing sufficient formaldehyde.....	0.0281	0.0003	0.0284
Sample containing insufficient formaldehyde.....	0.0211	0.0175	0.0386

Relations of Initial Purity of Milk to Development of Ammonia and Effect of Antiseptic.

A sample of "certified" milk which had been kept for seven days, much of the time at laboratory temperature, and which had become sour, still yielded when distilled without salt only 0.0004 per cent, and with salt only 0.0002 per cent of ammonia. On the other hand, a sample which had been contaminated with a few drops of stale separator slime,[1] was found after six days to yield without salt, 0.0084 per cent of ammonia. A duplicate sample of the same contaminated milk to which formaldehyde had been added in the proportion of 1:1000 yielded after six days (distilled without salt) 0.0010 per cent ammonia or only one-eighth as much as in the absence of the preservative.

The following indicates the effect of antiseptics as compared with spontaneous souring in a sample of exceptional purity.

In March, 1905, triplicate samples were taken of the mixed milk of a herd which was being managed with a view to the production of milk of the highest possible purity and cleanliness. One portion was treated with chloroform, three parts by weight of the latter in 100 parts of sample; the second received formalin to the extent of one part actual formaldehyde per 1000 parts of sample; the third portion received no preservative treatment whatever. The samples were kept in a room, the temperature of

[1] This was obtained from a separator through which only milk of good quality had been passed, but was allowed to become quite stale before using.

which averaged about 15° for three months, after which they were examined with the following results:

	Acidity*	Ammonia Found by Distillation. Without Salt. Per Cent.	With Salt. Per Cent.	Difference. Per Cent.
Portion with 3 per cent chloroform, 3 mos. old	10.0	0.0035	0.0026	0.0009
Portion with 0.1 per cent formaldehyde, 3 mos. old	2.8	0.0014	0.0007	0.0007
Portion with no preservative, 3 mos. old	22.0	0.0012	0.0001	0.0011

* Number of cc. of tenth-normal alkali required to neutralize 10 cc. of milk.

The portion which had been kept three months at room temperature with no preservative had a perfectly clean and not unpleasant sharp taste, somewhat resembling that of very sour kumyss. The portion to which formaldehyde had been added was not sour but had a slightly musty and rather nauseous taste. On testing it was found to still give a strong formaldehyde reaction.

SUMMARY.

Analyses of a large number of samples of mixed milk as sold in New York City showed an average of 0.0004 per cent of ammonia preformed at the time of examination together with an additional 0.0003 per cent of what is here called "cleavage" ammonia.

When ordinary milk is allowed to become stale the amounts of both preformed and "cleavage" ammonia usually increase.

Addition of 3 per cent of chloroform or 0.1 per cent of formaldehyde retards but does not stop proteolysis which results in the formation of ammonia. The production of those proteolytic products to which the "cleavage" ammonia is due appears to be retarded by these antiseptics to a greater extent than is the production of ammonia itself.

The greater the freedom from contamination the less apparent is the influence of the antiseptic upon the development of ammonia, and in a sample of exceptional purity spontaneous souring in the absence of preservative treatment appeared to inhibit the production of ammonia to a greater extent than did the addition of 3 per cent of chloroform or 0.1 per cent of formaldehyde.

The effects of pasteurization, and of antiseptics in amounts used as food preservatives, are being studied.

ON THE SEPARATE DETERMINATION OF ACETONE AND DIACETIC ACID IN DIABETIC URINES.

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(Received for publication, April 12, 1907.)

The Messinger-Huppert method is valuable for the determination of acetone and diacetic acid in urine, but the method gives only the sum of these two products; and there is manifest need of an additional quantitive method for the separate determination either of acetone or of diacetic acid.

Although acetone is a liquid with a boiling point of 56° C. and dissolves in water in all proportions, I have found that it can be removed from its solutions (even more readily than ammonia) by means of an air current and at ordinary room temperatures. The acetone can be determined in about half an hour according to the same principle and by the help of the same apparatus which I use for the determination of ammonia.[1] The determination is made as follows:

Measure 20–25 cc. of acetone solution or urine into an aërometer cylinder and add 0.2–0.3 gram of oxalic acid or a few drops of 10 per cent phosphoric acid, 8–10 grams of sodium chloride[2] and a little petroleum. Connect with the absorbing bottle (as in the ammonia determination) in which has been placed water and 40 per cent potassium hydroxide solution (about 10 cc. of the latter to 150 cc. of the former), and an excess of a standardized solution of iodine. Connect the whole with a Chapman pump and run the air current through for 20–25 minutes. (The air current should be fairly strong but not so strong as for the ammonia determination.) Every trace of acetone will now have been converted into iodoform in the receiving bottle. Acidify the contents of the latter by the addition of concentrated hydrochloric acid (10 cc.

[1] Folin: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 161, 1902.

[2] Acetone is insoluble in saturated sodium chloride solutions.

for each 10 cc. of the strong alkali used), and titrate the excess of the iodine, as in the Messinger method, with standardized thiosulphate solution and starch.

The determination of the acetone can be made simultaneously with the determination of the ammonia by the use of the same air current and even in the same sample of urine, but I do not recommend the last named combination except for cases where the amount of available urine is small.

Inasmuch as urines containing acetone and diacetic acid are also the urines in which clinicians determine the ammonia, the following combination of acetone and ammonia determinations will probably prove practical and time-saving:

The ammonia determination is started first in the usual manner, except that I make use of an ordinary Chapman pump instead of the more rapid but not at all necessary air blast which I ordinarily use for ammonia determinations. A second cylinder and absorption apparatus are then arranged as described above for the acetone determinations and connected with the cylinder in which the ammonia is being determined. The air current is then regulated for 20–25 minutes with special reference to the acetone determination. The acetone apparatus is then disconnected and the acetone determination finished as usual. Thus the air current need never be stopped and the ammonia determination is hardly if at all interfered with by the acetone determination.[1]

[1] In this connection I may be permitted to state that in my opinion no other method yet devised for the determination of ammonia is so accurate for all kinds of urine as my air current method. An excellent alternative method is the vacuum distillation method as described by Shaffer. *Amer. Journ. of Physiol.*, viii, p. 348, 1903.

The vacuum distillation method has an interesting history. It was originally described by Boussingault in 1850, but was criticised out of existence by the leading German physiological chemists of his time. In recent years many have made use of the method in one way or another, but no one except Shaffer has shown any disposition to rectify the error committed against Boussingault. The vacuum distillation method is as truly Boussingault's as the desiccator method is Schlösing's. In the current literature the method is accredited to Wurster; Nencki, and Zaleski; Söldner; Steurer, Krüger and Reich; Krüger, Reich and Schittenhelm, etc., but never to its real author. Recently Schittenhelm has even complained because the "Krüger-Reich-Schittenhelm Methode" has not received recognition!

By combining this method with the usual Messinger-Huppert method I have recently made a number of determinations in diabetic urines. The urines were obtained from Dr. Elliott P. Joslin, and were only a few hours old when taken for the acetone determinations. The series given below may be cited as illustrations of the acetone and diacetic acid contents of such urines:

	1	2	3	4	5	6	7
	2200 cc.	1980 cc.	3600 cc.	1990 cc.	4750 cc.	1370 cc.	520 cc.
Acetone in 20 cc.	3.15 mg.	5.0 mg.	7.1 mg.	4.8 mg.	5.6 mg.	4.3 mg.	2. mg.
Total	.35 g.	.49 g.	1.28 g.	.48 g.	1.23 g.	.29	.05 g.
Acetone + diacetic acid in 20 cc.	13.5 mg.	13.8 mg.	38.8 mg.	15.8 mg.	29.6 mg.	16.7 mg.	20.5 mg.
Total	1.48 g.	1.36 g.	10.2 g.	1.57 g.	10.2 g.	2.29 g.	.53 g.
Total NH_3		3.93 g.	5.05 g.	2.47 g.	5.7 g.	1.53 g.	.87 g.

For the sake of convenience I have in the above table considered 1 cc. of tenth-normal iodine solution equal to 1 mgm. of acetone, whereas it actually corresponds to only $\frac{58}{60}$ mgm. Urine No. 6 represents only a twelve hour quantity of urine at the beginning of a lethal attack of diabetic coma. Urine No. 7 represents the following complete twenty-four hour urine from the same patient.

It will be seen that in my acetone titrations I have been dealing with rather small quantities. On the other hand I am confident that in no single case did the error in the determination exceed 0.1 cc. of tenth-normal iodine. Such an error in urine No. 7 is indeed five per cent of the total, yet the absolute error for the whole 24-hour quantity amounts to less than 3 mgm. The same error in urine No. 5 where the total volume of urine is 4750 cc. would amount to less than 2 per cent of the total, *i.e.*, about 24 mgm.

The values recorded in the above table are not uninteresting because they represent the first definite information yet obtained concerning the relative proportions of acetone and diacetic acid in diabetic urines. In this paper I wish, however, to consider those figures only from an analytical standpoint.

The acetone values vary from about one-third to less than one-tenth of the total values given by the Messinger-Huppert method. In the light of this fact it is of course important that the acetone should be determined under conditions involving the least possible decomposition of the diacetic acid. Decomposition of 5 per cent of the diacetic acid may mean an error of 50 per cent in the acetone determination. The relatively great preponderance of the diacetic acid points also to the necessity or importance

of making the acetone determinations direct and independent of any Messinger-Huppert distillations. In a differential method based on distillation before and after the removal of the acetone by the air current an error of 5 per cent in each distillation might mean an error of more than one hundred per cent in the acetone determination. I emphasize these facts because the method described above is capable of many variations and modifications.

Future experience may show the necessity of using 35 or even 50 cc. of urine or other acetone solutions for the acetone determinations; and I believe that the method will be found equal to such an emergency. But I scarcely believe that more than 50 cc. can be used in the case of solutions which contain not only acetone but also diacetic acid. The following determinations of acetone made in a pure acetone solution indicate the possibilities of the method in this direction:

(1)	20 cc.	acetone	solution +	100 cc.	H_2O =	by direct	titration			23.95 cc.	$\frac{N}{10}$ I.
(2)	"	"	"	100	"	"	"	"	"	23.95	"
(3)	"	"	"	100	"	"	"	air current	(20 min.)	23.8	"
(4)	"	"	"	100	"	"	"	"	25 "	23.8	"
(5)	"	"	"	100	"	"	"	"	20 "	23.9	"
(6)	"	"	"	100	"	"	"	"	25 "	23.8	"
(7)	"	"	"	30	"	"	"	"	25 "	23.4	"
(8)	"	"	"	30	"	"	"	"	35 "	23.6	"

In the experiments 7 and 8 the solutions in the cylinders were rinsed out and the acetone remaining determined by direct titration. No. 7 showed 0.5 cc. tenth-normal iodine, No. 8 showed 0.2 cc. tenth-normal iodine equivalent of acetone.

Schwarz who several years ago attempted to use air currents for the removal of acetone from urine found that it required twelve hours to remove 50 mg. from 100 cc. of liquid.[1]

Schwarz barely missed discovering the efficiency of air currents for this purpose. He worked not only with large volumes of liquid (100 cc.), but also with small volumes (20 cc.). Having, however, once found that a twelve hour current was necessary in the case of 100 cc. he did evidently not allow himself to hope that anything less than several hours could suffice for 20 cc.[2]

The method described in this paper is not free from possible sources of error and these should be clearly understood in order to guard against mistakes that would certainly come in and render the results unreliable.

[1] *Arch. f. exp. Path. u. Pharm.*, xl, p. 189, 1898.

[2] See Waldvogel's monograph on Die Acetonkörper, p. 33, 34, 1903.

The spontaneous decomposition of diacetic acid is not the only circumstance which makes a rapid acetone determination necessary. A fact, at least equally important, is the rapid spontaneous decomposition of the alkaline hypoiodite solution used for the decompositon of the acetone. Schwicker's[1] statement that alkaline hypoiodite solutions are almost completely converted into iodate solutions in the course of 30 minutes is not quite in accordance with the facts. Such solutions can be kept for an hour or longer and will still give considerable precipitates when acetone is added. For practical analytical purposes Schwicker's statement may, however, be considered substantially correct. Since the iodate is of no use for the formation of iodoform it is clear that it is of the greatest importance that the acetone should be driven from the urine into the alkaline iodine solution as rapidly as possible, keeping in mind at the same time that there is also a limit to the rapidity with which the alkaline iodine solution can take up the acetone.

These difficulities are at their minimum—become perhaps almost negligible—in actual urine work where the amount of acetone involved is, as I have shown, very small. But the danger is there, and anyone wanting to make acetone determinations should be aware of it.

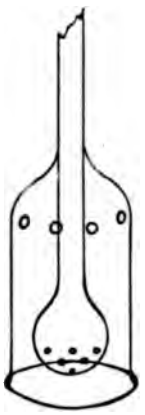
It goes without saying that no one should attempt to make acetone determinations in urines or other unknown solutions until he has learned to know his air current and his apparatus by working with known acetone solutions. Such a solution can be made and standardized (by direct titration) in the course of a few minutes. Ten cc. of acetone diluted to one-fourth of a liter, and 20 cc. of this solution diluted to half a liter makes a suitable test solution of acetone.

In working with such a solution the following hints may be of service: (1) The excess of standardized iodine solution should not be too small (I use an excess of 10–15 cc.). (2) No time should be wasted after the strong caustic potash solution has been added to the diluted iodine solution in the absorption bottle. (3) If the analytical figure obtained is too low the air current has been either too fast for the absorption apparatus, or it has been so slow as to give time for the loss of too much hypoiodite. A second determination by means of a slower air current will show which is the case. A still lower result indicates that too slow a current was used in the first experiment and the work must be repeated with a stronger air blast. (4) The completeness with which the air current removes the acetone from its

[1] Cited from Neubauer u. Vogel, *Harnanalyse*, 10th ed., p. 761.

solutions can best be determined by rinsing the solution into a beaker and testing with the alkaline iodine solution. A twenty-minute air current should remove the acetone from 20 cc. of its solution so completely that the subsequent addition of iodine and alkali fails to give an appreciable test for acetone. (5) The caustic potash added to the iodine solution must be free from nitrites. The presence of nitrites and nitrates is revealed by the reappearance of the blue color after the titration is seemingly finished.

A freshly prepared, strongly alkaline solution of hypoiodite is at least as effective an absorbent of acetone as is a dilute solution of acid for the absorption of ammonia. But as the acetone comes over (and must come over) more rapidly than does the ammonia under similar conditions, it is



important that adequate provision be made for a thorough contact of the air carrying the acetone, with the iodine solution. The double absorption tube which I use in connection with the ammonia determinations[1] is satisfactory in this case also, except that the rubber stopper cannot be used. Alkaline hypoiodite solutions act on rubber. The two parts of the tube must therefore be sealed together with the blast lamp.[2] The cut given above makes further description of this tube unnecessary. The only special point to be observed in its construction is that the holes in the outer tube should be decidedly larger than the holes in the bulb of the inner tube.

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[1] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 169.

[2] I have obtained some excellent tubes thus sealed together from Eimer and Amend, New York.

IV. RESEARCHES ON PYRIMIDINS: ON A COLOR TEST FOR URACIL AND CYTOSIN.

PLATE II.

(Twenty-first Paper.)

By HENRY L. WHEELER AND TREAT B. JOHNSON.

(*From the Sheffield Laboratory of Yale University.*)

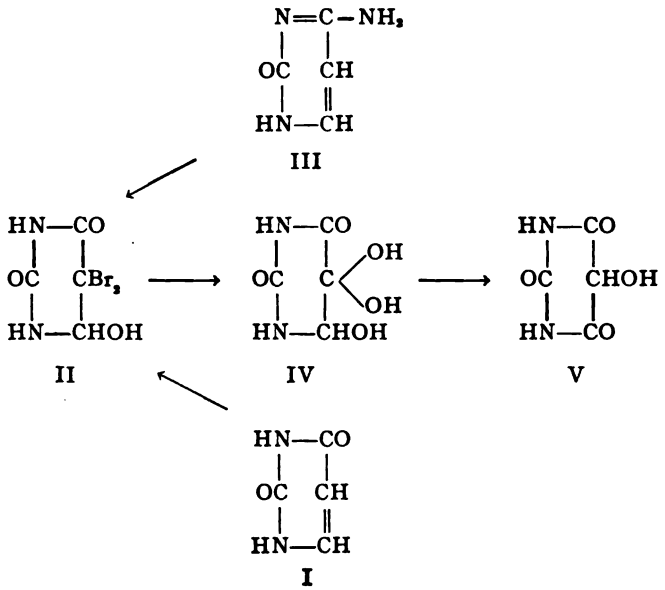
(Received for publication, April 19, 1907.)

When uracil or cytosin is dissolved in bromine water and the solution is treated with an aqueous solution of barium hydroxide in excess, a purple or violet-blue precipitate or color is produced even in dilute solutions.

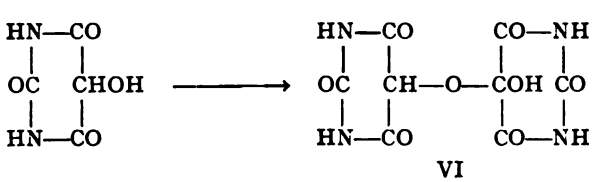
The formation of the purple precipitate involves several intermediate reactions that are explained in the following manner: Uracil (I) and bromine water first react to form dibromoxyhydrouracil (II), and the same compound is also obtained when cytosin (III) is treated with bromine water. Dibromoxyhydrouracil is very sensitive toward alkalies. When treated at ordinary temperature with an excess of barium hydroxide the two atoms of bromine are replaced by hydroxyl groups and isodialuric acid (IV) is formed. Isodialuric acid then undergoes a rearrangement into dialuric acid (V).[1] Both isodialuric and dialuric acids give a violet-blue precipitate with barium hydroxide as observed by Behrend and Roosen.[2]

[1] Behrend and Köch: *Ann. d. Chem.* (Liebig), cccxv, p. 246, 1901.

[2] *Ann. d. Chem.*, (Liebig), ccli, p. 244, 1889.



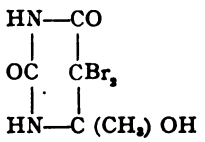
That the present test involves the formation of dialuric acid was shown as follows: The freshly prepared barium precipitate was dissolved in hydrochloric acid and the barium was removed by means of dilute sulphuric acid. On evaporating this solution then in a desiccator we obtained crystals of alloxantin (VI). Dialuric acid undergoes oxidation in the air to alloxantin,[1] while such behavior was never observed in the case of isodialuric acid.[2]



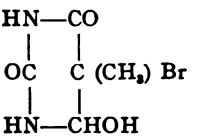
Behrend was the first to show that certain pyrimidins give bromoxyhydro-derivatives. For example, he prepared dibromoxyhydromethyluracil (VII)[3] from 4-methyluracil.

[1] Baeyer: *Ann. d. Chem.* (Liebig), cxxvii, p. 12, 1863.
[2] Behrend and Roosen: *Loc. cit.*
[3] *Ann. d. Chem.* (Liebig), ccxxix, p. 18, 1885.

This compound gives no color with barium hydroxide. Of more interest in connection with the test, however, is the fact that the similar compound from thymin, bromoxyhydrothymin (VIII), described by Walter Jones,[1] also gives no color with barium hydroxide. Obviously these compounds would not be expected to yield dialuric acid on treating with baryta water.

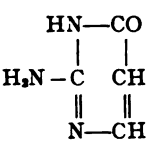


VII

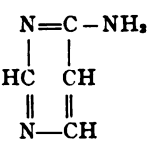


VIII

In Richard Burians' interesting work[2] on the question whether cytosin is a primary product or whether it results by secondary decomposition of some other substance when the nucleic acids are submitted to hydrolysis, he boiled guanin and adenin mixed with various carbohydrates in 30–40 per cent sulphuric acid. He did not obtain cytosin by this treatment, but instead, from guanin 2-amino-6-oxypyrimidin (isocytosin) was formed (IX).[3] The synthesis of this pyrimidin has been described by us.[4] On the other hand, adenin (5 grams) gave 6-aminopyrimidin (X),[5] (0.5 gram). These pyrimidins are therefore to be considered in the test.



IX



X

When isocytosin is treated with bromine water it yields a bromine derivative that is not identical with dibromoxyhydrouracil. This substance gives an intense blue color on carefully adding a solution of barium hydroxide. It is a more decided blue

[1] *Zeitschr. f. physiol. Chem.*, xxix, p. 20, 1900.
[2] *Ergeb. d. Physiol.* (Asher-Spiro), v, p. 794, 1905.
[3] *Amer. Chem. Journ.*, xxix, p. 492, 1903.
[4] Büttner: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2232, 1903.
[5] Wheeler and Bristol: *Amer. Chem. Journ.*, xxxiii, p. 458, 1905.

than that which results from dibromoxyhydrouracil and, what is more important, it is readily distinguished from the latter by immediately disappearing on adding an excess of the barium hydroxide solution. This behavior serves as a delicate test for isocytosin.

Finally 6-aminopyrimidin was prepared by a new method; starting with 2-thiouracil,[1] which can readily be obtained in quantity, 2,6-dichlorpyrimidin was prepared by means of phosphorous pentachloride.[2] The dichlorpyrimidin then gave 2-chlor-6-aminopyrimidin with alcoholic ammonia, and this was found to reduce smoothly to 6-aminopyrimidin when warmed with concentrated hydriodic acid.

The material thus obtained gave no color whatever with bromine water and barium hydroxide.

THE TEST.

Bromine water is added to about 5 cc. of the solution to be examined until the color is permanent. Too much bromine is to be avoided since a large excess interferes with the test.

It is advisable, especially when only small quantities of cytosin or uracil are present to remove the excess of bromine by passing a stream of air through the solution. Then on adding barium hydroxide in excess the purple color is almost immediately produced.

Very dilute solutions do not give the test. In such cases on evaporating to dryness and then taking up the material in a little bromine water, removing the excess of bromine, etc., a quantity as small as 0.001 gram of uracil gives a decided bluish-pink or lavender color.

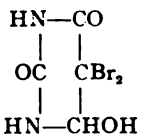
In applying the test in the case of cytosin it is advisable to warm or boil the solution with bromine water, cool, and then apply the test as above, being sure to have a slight excess of bromine present before adding barium hydroxide. Dibromoxyhydrouracil is decomposed by prolonged boiling with water into

[1] Gabriel: *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 1690, 1905; Johnson and Menge: This *Journal*, ii, p. 115, 1906.

[2] Gabriel: *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 1690, 1905.

5-bromuracil,[1] which gives no color with barium hydroxide. If, however, 5-bromuracil is treated with bromine water it is converted back again into dibromoxyhydrouracil. Picric acid interferes with the color and should be removed before applying the test.

Dibromoxyhydrouracil,



In preparing this compound for use in further experiments we usually took 5 grams of uracil, suspended in 20 cc. of water, and added a little over 15 grams of bromine. The uracil dissolved completely on warming, and, on cooling, a crystalline mass separated. The material thus obtained had a yellow color from excess of bromine, and the yield that first separated was almost 90 per cent of the calculated. On crystallizing once from water colorless, large, flat prisms or blocks separated. The habit of these crystals is shown in the microphotographs (magnified 60 times) Plate II.

The same substance was obtained when 0.6 gram of cytosin sulphate was suspended in water and bromine added until the salt dissolved. The solution was then concentrated to a small volume and cooled. The prisms obtained melted at 205–6° C. (Analysis (III).

The analytical results were as follows:

	Calculated for $C_4H_4O_2N_2Br_2$:	Found: I.	II.	III.	IV.
N.....	9.72	9.59	9.63	9.46	
Br.....	55.55				56.00

Dibromoxyhydrouracil melts with effervescence at 203–6°. It shows signs of decomposition below this temperature. It is more soluble in water than uracil. The solution of the pure white crystals is neutral to litmus, but on boiling it has an acid reaction and finally 5-bromuracil separates. In accordance with this silver nitrate gives no precipitate in the cold but on warming with this reagent silver bromide separates.

[1] Wheeler and Merriam: *Amer. Chem. Journ.*, xxix, p. 486, 1903.

Dibromoxyhydrouracil dissolves readily in alcohol. If boiled with alcohol 5-bromuracil separates. If the alcoholic solution is treated with a solution of sodium in alcohol a purple percipitate is at once produced similar to the barium hydroxide percipitate. Alcoholic potassium hydroxide also produces a similar colored precipitate. These colored alkali salts differ from the barium hydroxide precipitate by being instantly decomposed and decolorized by treatment with water. The aqueous solution then turns green and finally orange on standing.

Aqueous ammonia immediately dissolves the dibrom-derivative, and removes bromine; the solution slowly takes on yellow, then a garnet color and if sufficient material is present a reddish-brown precipitate separates.

Dibromoxyhydrouracil is almost insoluble in ether.

THE PURPLE PRECIPITATE.

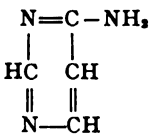
The precipitate produced by adding barium hydroxide to an aqueous solution of dibromoxyhydrouracil when exposed on paper to dry in the air turned red. When treated with acetic acid it changed to a bright red powder, while the precipitate when freshly precipitated dissolved completely in acetic acid. The analysis of the precipitate was therefore abandoned. It was shown that the substance yields alloxantin on treatment with acids as follows: Four and a half grams of dibromoxyhydrouracil were dissolved in 40 cc. of water and added to 11 grams of crystallized barium hydroxide in 100 cc. of water. The purple precipitate was rapidly filtered but no attempt was made to wash it. It was immediately dissolved in dilute hydrochloric acid and the barium was removed by adding 20 per cent of sulphuric acid. The colorless solution, which on testing a portion with barium hydroxide again gave a purple precipitate, was allowed to evaporate in a desiccator. On standing over night it gave small, stout, colorless transparent prisms. These melted at 243° C. with effervescence. Behrend and Friedrich[1] state that alloxantin melts at 243–5°. This material was not dialuric acid since its aqueous solution failed to decompose carbonate of sodium and it was not

[1] *Ann. d. Chem.* (Liebig), cccxliv, p. 11, 1906.

isodialuric acid because it melted over 100° higher. A nitrogen determination agreed with the calculated for alloxantin:

	Calculated for $C_8H_6O_8N_4+2H_2O$:	Found:
N.....	17.40 per cent.	17.11 per cent.

6-Aminopyrimidin,



One gram of 2-chlor-6-aminopyrimidin was dissolved in 20 cc. of colorless, concentrated hydriodic acid and then evaporated to dryness on the water bath. Iodine separated in abundance. The residue was evaporated several times with a solution of sulphur dioxide. The colorless solution was then treated with an excess of silver sulphate, filtered and the silver was then removed with hydrogen sulphide. On concentrating the solution a syrup was obtained, which, when taken up in boiling alcohol, gave well-crystallized colorless prisms. The yield was over 0.8 gram. The crystals melted at 143° to clear oil, and nitrogen determinations agreed with the calculated for an acid sulphate of 6-aminopyrimidin.

	Calculated for $C_4H_5N_3.H_2SO_4$:	Found: I.	II.
N.....	21.76	21.55	21.11

The solution of this material was freed from sulphuric acid by means of barium hydroxide and the excess of barium hydroxide was removed with carbonic acid. The free base proved to be extremely soluble in water. The aqueous solution was precipitated by phosphotungstic and picric acid, and it gave a precipitate of silver salt when treated with silver nitrate in neutral solution. This precipitate was soluble in ammonia. With bromine water and barium hydroxide it gave no color.

ON THE RELATIVE EFFICIENCY OF THE VARIOUS METHODS OF ADMINISTERING SALINE PURGATIVES.

By FRANK W. BANCROFT.

(*From the Rudolph Spreckels Physiological Laboratory of the University of California.*)

(Received for publication, April 10, 1907.)

In a recent paper Auer[1] has come to the conclusion "that the subcutaneous and intravenous injection of magnesium sulphate and chloride, sodium sulphate, phosphate, and citrate does not produce purgation in rabbits." These results are just the reverse of those arrived at by MacCallum in this laboratory for all these salts except magnesium chloride. Since I had the opportunity of seeing many of the experiments of Dr. MacCallum, and since his death prevents him from stating his methods and results in greater detail and from pointing out why Auer has arrived at different results, I have attempted to supply these deficiencies. Accordingly, I have repeated some of MacCallum's experiments to obtain the quantitative data which he did not consider it worth while to publish, and have also made some additional experiments in which the effects of dosage by mouth and subcutaneously were compared.

TERMS AND METHODS.

Obviously in an investigation of this kind the criterion of purgation is of the first importance. Auer[2] says "by purgation is here understood the passage of soft and unformed feces in amounts exceeding that which normal animals might conceivably pass." In accordance with this definition Auer does not consider that mere increase in the amount of the feces without an increase in fluidity constitutes purgation. MacCallum, on the other hand, considers that both increase in amount and in fluidity

[1] *Amer. Journ. of Physiol.*, xvii, p. 15, 1906.
[2] *Ibid.*, p. 17.

of the feces should be taken into consideration in estimating whether purgation has taken place. This difference is partly responsible for the difference in the conclusions of the two authors. Now in this connection it should be pointed out that, as in the rabbit, the feces are usually dry and well formed, and since the action of small doses of weak purgatives is to increase only the *amount* of feces eliminated in a given time, it is essential in such investigations that the *amount* of the feces should be carefully considered.

Another and more important difference in the method of experimentation of the two investigators concerns the nature of the control experiments. This difference, I think, is mainly responsible for the difference in conclusions. Auer seems in general not to have kept control animals for each experiment. For, in his paper, it is only on February 15 that he compares the feces of experimental animals with the feces of control animals observed at the same time. In general he seems to have depended for his controls on animals which were not observed at the same time as the experimental animals with which they were compared; and which therefore may have been in a very different condition from that of the experimental animals. Furthermore he does not appear to have attached much weight to the controls which he did keep. Thus, when the feces of the experimental animals are slightly greater in amount or fluidity than the average of his controls he does not conclude that the salts injected have had a slight effect, but seems to think that, as indicated by his definition of purgation, the purgative effect of the salts has been proved only when the result produced is above the maximum of the controls. MacCallum's method of experimentation, when the actual passage of feces was to be observed, was quite different.[1] He always kept controls which were observed *at the same time* as the animals experimented upon, and which were treated exactly like the experimental animals in every respect except that no salts were injected into them. The feces passed by the controls and the experimental animals were compared. It was found that

[1] MacCallum: *Amer. Journ. of Physiol.*, x, p. 103, 1904; *On the Mechanism of the Physiological Action of the Cathartics. Univ. of Cal. Publications:* Berkeley, The University Press, pp. 11–13, 1906.

the experimental animals passed more feces, and from this it was concluded that purgation had taken place, and that the purgation had been caused by the salts injected. When, in addition, it was found that the feces eliminated by the experimental animals were more fluid than those eliminated by the controls, this fact was also taken to mean that purgation had taken place.

The necessity for simultaneous controls is seen when it is remembered that all that the purgatives can do, is to increase the rate with which the feces already present are eliminated. The milder purgatives will cause only an elimination of the feces already present in the large intestine; while the more powerful ones will in addition bring about peristalsis in the caecum and the elimination of fluid feces. Now, as Auer used only small doses of the salts which MacCallum had classed among the weaker purgatives the need for adequate control experiments was even greater than usual. For, the nature of the food, the amount eaten, the time after eating and drinking, exercise, etc., may all affect the amount and nature of the feces in the large intestine. To determine, therefore, whether a small dose of a weak purgative is having any effect it is essential that the animals compared should start with about the same amount of feces in their large intestine, and the only way to insure this is to take similar animals and to treat them alike.

EXPERIMENTAL RESULTS.

My experiments fall naturally into two groups:

1. Those which simply repeated MacCallum's experiments, small doses being used.

2. Those in which the largest possible doses were used, in order to obtain fluid feces.

At the outset I may say that MacCallum's results have been confirmed in every respect. As an example of the experiments where small doses were employed the following may be taken:

Experiment 1. Four rabbits of about the same size and weight were confined in four similar cages. The animals were separated into two pairs. In one pair the same rabbit was always the control, in the other pair one animal was control one day, and the experimental animal on the next. The rabbits had been fed on hay, grain, and vegetables, but during the

experiment were fed only on carrots and water, one half of each carrot going to each member of a pair. The purgative used was $\frac{M}{6}$ sodium citrate, which was injected subcutaneously into the dorsal region in the amounts specified in the table. The feces were collected in jars with false bottoms of wire mesh, so that they were never soaked by the urine.

The most significant thing about these results is that *the amount of feces passed during the first 3 to 5 hours after the injection of the citrate, in every case, was more than that passed by the control animal.* This happened even when the total 24-hour feces of the control animal were more than those of the experimental one. The total amount of feces passed by all the experimental animals during these first 3 to 5 hours after injection was 109.33 grams, while that passed by the controls during the same period was only 4.63 grams. Thus, the amount eliminated by the injected animals during these first few hours was 23 times that eliminated by the controls.

Another noticeable effect of the citrate is seen in comparing Rabbits 1 and 2 which were injected on alternate days. In this case the total daily amount was greater for the injected animal on every day except one. The sum of the totals for the injected Rabbits was 156.83, or about 4 times as much as the sum of the totals for the controls (38.26). For quite a while the rabbits passed almost all their feces on alternate days; and that this alternation was due to the citrate and not to accident is indicated by the fact that it immediately disappeared on the fifth and tenth days when no citrate was given. It is evident that a period of constipation follows the purgation due to the citrate.

But although these results appear so clear and convincing, it is still probably worth while to spend some time in pointing out that it is possible by means of an uncritical examination, to make it appear that they mean little or nothing. Thus, when the total amounts or averages of Nos. 3 and 4 are compared they are found to be practically equal. From this it might be concluded that the citrate had had no effect; and if the feces had been weighed only once a day there would have been no escape from this conclusion. What the citrate has done is not to *make* feces, but to *accelerate* their elimination for a few hours after the injection. After that the control animal may catch up again, as happened with both controls on the first day. The total amount of the feces depends.

TABLE I. EXPERIMENT I.

Day.	Hour.	Rabbit 1.		Rabbit 2.		Rabbit 3.		Rabbit 4.	
		Dose of Sodium Citrate.	Feces.	Dose of Sodium Citrate.	Feces.	Dose of Sodium Citrate.	Feces.	Dose of Sodium Citrate.	Feces.
		cc.	gm.	cc.	gm.	cc.	gm.	cc.	gm.
1	12:00 m.	0		15		0		30	
	Next 3¾ hrs.		2.6		24.2		0.0		16.6
	Next 20¼ hrs.		22.65		12.5		30.83		16.93
	Total 24 hrs.		25.25		36.7		30.83		33.53
2	2:05 p. m.	30		0		0		30	
	Next 3 hrs.		7.74		0.0		0.0		5.7
	Next 16 hrs.		18.7		0.0		30.65		22.76
	Next 5 hrs.		0.0		0.0		0.64		0.0
	Total 24 hrs.		26.44		0.0		31.29		28.46
3	2:15 p. m.	0		30		0		30	
	Next 3 hrs.		0.0		7.6		0.0		7.44
	Next 15 hrs.		0.2		21.7		0.84		0.75
	Next 6 hrs.		0.0		3.34		0.06		5.5
	Total 24 hrs.		0.2		32.64		0.90		13.69
4	2:16 p. m.	30		0		0		30	
	Next 3 hrs.		3.55		1.76		0.0		2.55
	Next 18 hrs.		4.9		0.10		0.0		6.77
	Next 4 hrs.		0.05		0.0		0.0		1.3
	Total 25 hrs.		8.50		1.86		0.0		10.62
5	Next 19 hrs.		0.05		0.10		0.0		0.0
6	10:00 a. m.	0		30		0		30	
	Next 3 hrs.		0.0		0.14		0.17		7.58
	Next 21 hrs.		10.5		0.03		0.0		6.6
	Total 24 hrs.		10.5		0.17		0.17		14.18
7	11:00 a. m.	30		0		0		30	
	Next 3 hrs.		1.9		0.0		0.0		4.8
	Next 18 hrs.		19.35		0.0		32.29		0.0
	Total 21 hrs		21.25		0.0		32.29		4.8
8	9:10 a. m.	0		30		0		30	
	Next 5 hrs.		0.0		13.43		0.0		5.2
	Next 19 hrs.		0.3		13.4		8.2		0.2
	Total 24 hrs.		0.3		26.83		8.2		5.4
9	10:20 a. m.	30		0		0		30	
	Next 4 hrs.		0.3		0.1		0.0		0.6
	Next 25 hrs.		4.4		0.05		7.8		9.9
	Total 29 hrs.		4.3		0.15		7.8		10.5
10	Next 26 hrs.		0.6		0.1		11.2		3.2
	Totals		97.39		98.55		122.68		124.38
	Average 24 hrs.		9.74		9.85		12.27		12.44

in the first place, upon the amount and the bulkiness of the food eaten; and only secondarily on the amount of water in the feces. It is only by increasing the water that moderate doses of purgatives can increase the total amount of feces eliminated in an experiment of several days' duration. We should expect, however, that the amount of water in the feces should be influenced by the citrate, and the reason why the results do not show it is because No. 3 for some unknown reason had the diarrhoea. After the first day the feces of all the rabbits were always unusually moist whenever they were eliminated in considerable quantities. This may have been due to the citrate in Nos. 1, 2 and 4, and to some other unknown cause in No. 3; or it may have been due to the carrots in all four rabbits. At any rate our results do not allow us to conclude that the citrate has increased the amount of water in the feces. In later experiments, however, it will be shown that subcutaneous injection of saline purgatives does produce more fluid feces.

A further consideration of the rate of the elimination of feces will show how absolutely essential it is to have adequate control animals. The total amount of feces eliminated by all four rabbits during the 240 hours of observation was 443.0 grams, giving an average rate of 0.46 grams per rabbit per hour. The 109.33 grams eliminated by the injected animals during the first 3 to 5 hours after the injections were eliminated in 27.75 hours; which gives an hourly rate of 1.97 grams per rabbit. Were it not for the controls we would be forced to conclude that the citrate had accelerated the elimination only about 4 times, while a comparison with the controls shows us that the rate has been accelerated 23 times. The explanation appears to be that normally the feces are not passed at a uniform rate, but much more rapidly at certain times of day than at others; and the time of day at which the injections were made was one during which ordinarily but little feces would be passed. But this would not have been realized if adequate controls had not been kept.

Finally, the last thing in the results to which I wish to call attention is the effect of the food on the amount of the feces. Before the experiment was begun the rabbits had been fed largely on hay, which contains much more cellulose than the carrots fed during the experiment. The cellulose is not digested and con-

stitutes the main bulk of the feces. So we should expect much less feces on a diet of carrots than on a diet composed largely of hay. The evident decrease in the daily elimination during the course of the experiment is due to this change of diet.

The effect of the character of the food on the amount of feces passed, and so indirectly upon the purgation is strikingly shown when the results just discussed are compared with those of the next experiment.

Experiment 2. The same rabbits as in Experiment 1, but they have been fed hay, grain, and water for several days. The same diet is continued during the experiment. $\frac{M}{6}$ sodium citrate is given to No. 1 by mouth, and to No. 2 subcutaneously.

TABLE II. EXPERIMENT 2.

Day.	Hour.	No. 1, 1580 gm. per os.		No. 2, 1501 gm. subcut.		No. 3. 1697 gm. Control.	No. 4. 1621 gm. Control.
		Dose of Sodium Citrate.	Feces.	Dose of Sodium Citrate.	Feces.	Feces.	Feces.
		cc.	gm.	cc.	gm.	gm.	gm.
1	2:10 p. m.	30		30			
	First 1 hr.		4.9		13.8	8.3	2.4
	First 3 hrs.		13.8		21.6	14.9	5.9
	Next 15½ hrs.		17.4		46.5	57.4	19.5
	Total 18½ hrs.		31.2		68.1	72.3	25.4
2	9:20 a. m.	30		30			
	First 1 hr.		23.1		0.0	2.3	4.3
	First 4½ hrs.		27.4		8.7	23.8	5.5
	Next 23½ hrs.		2.2		23.9	66.7	35.3
	Total 28 hrs.		29.6		32.6	90.5	40.8
3	1:40 p. m.	30		30			
	First 1 hr.		13.6		8.8	7.8	8.8
	First 4 hrs.		23.9		18.2	24.8	18.1
	Next 16 hrs.		29.1		0.5	36.2	32.0
	Total 20 hrs.		53.0		18.7	61.0	50.1
4	10:00 a. m.	30		30			
	First 1 hr.		2.8		10.0	2.1	13.4
	First 4 hrs.		14.6		21.0	16.1	32.9
	Next 24 hrs.		69.8		30.4	62.3	60.1
	Total 28 hrs.		84.4		51.4	78.4	93.0
	Totals						
	First 1 hr.		44.4		32.6	20.5	28.9
	First 3–4½ hrs.		79.7		69.5	79.6	62.4
	Daily Totals		198.2		170.8	302.2	209.3

In this experiment I used the same rabbits employed for Experiment 1, but the diet had increased the average daily feces from 11.1 grams in Experiment 1 to 54.4 grams. This additional amount of feces almost entirely obliterated any effect that might be due to the citrate. It is only by comparing the totals that it can be seen that in spite of the fact that the controls are larger animals, and pass more feces, yet during the first hour after the administration of the citrate the experimental animals passed the greater amount of feces. It should be noted, however, that there is no great difference, in this case, between the effects of the citrate when given by mouth or subcutaneously. But this diminution in the effect of the purgative with the more bulky diet should not surprise us. The rabbits are now constantly passing pellets. On one day hourly observations were made on all four rabbits from 10:00 a. m. to 4:00 p. m., and there was only one hour for one of the rabbits during which no feces were passed. On the average a pellet was passed every 6 minutes throughout the 24 hours, and with such an output it is not surprising that the effects of small doses of sodium citrate might be hard to detect. But that decided purgation is possible, even on a diet of hay and grain, will be shown in the following experiments in which much larger doses were given.

EFFECTS OF MAXIMAL DOSES.

In this second series the attempt was made to obtain fluid feces. The food given was usually hay and grain. In some cases other food was given, but in every case the same food was given to both control and experimental animals. Sodium citrate, sodium sulphate, and barium chloride were given both by mouth, subcutaneously, intravenously, and intra-abdominally. It was found possible to obtain fluid feces by means of all four methods of administration. They could be obtained in most cases when the purgatives were given by mouth; but the doses required were enormous. When, however, the same doses were given subcutaneously or intravenously the rabbits were often killed before fluid feces were obtained. In these fatal cases post mortems showed that the salts had acted as purgatives, but that their action had been cut short by death.

EFFECTS OF SODIUM CITRATE.

The above statements were particularly true for this salt. Divided doses of from 50 to 120 cc. of $\frac{M}{6}$ sodium citrate given subcutaneously or intra-abdominally killed the rabbits in from 3 to 9 hours, according to the rapidity with which the solutions were injected. When given by mouth 100 to 250 cc. given at the rate of about 50 cc. per hour produced fluid feces in about one-third of the experiments. When the citrate was given in other ways than by mouth the rabbits survived a dose of 100 cc. or more in only one case. In this case 180 cc. were given, and, as was to be expected, it had the same effect as when given by mouth; causing after 6 hours the elimination of perfectly fluid and unformed feces in considerable quantities. In this experiment the animal that was treated by mouth received 250 cc. of citrate during 5 hours, but produced only pasty and sticky feces that were still formed. The feces of the control were dry and hard.

Post mortems were made on the animals that died, and in almost every case it was evident that if the intestines had continued to function for a little longer the fluid feces which had replaced the formed pellets usually found in the large intestine would have been extruded. It is thus seen that while it is much easier to obtain fluid feces by giving sodium citrate by mouth than by giving it subcutaneously or intra-abdominally this difference in results is due to the increase in toxicity when given in these latter ways, and not because the salt has a different action on the digestive tract.

EFFECTS OF SODIUM SULPHATE.

In these experiments, also, the main attempt was to obtain fluid feces, and the experiments were not arranged to show the greatest differences in the weight of the feces of control and experimental animals; but in spite of this disadvantage, striking quantitative results were obtained. These results are shown in the following summaries:

These summaries show that in spite of the fact that the injected animals got such large doses that they were seriously injured and often killed by them, thus of course diminishing the activity of

the digestive tract, the injected animals passed on the whole much larger quantities of feces than the controls. Thus, the rabbits that were injected subcutaneously passed about 3½ times as much feces as the controls; while those that were injected intravenously passed about 10 times as much. These results

TABLE III.

SUMMARY OF SUBCUTANEOUS INJECTIONS OF SODIUM SULPHATE.

Date.	Duration of Experiment.	Dose of Na_2SO_4.	Weight of Feces in Grams.		
			Control.	Dose by Mouth.	Dose subcut.
Jan. 1	19 hrs.	110 cc. m/2	18.6	8.3	62.4
" 7	20 "	80 cc. m/2	0.5	56.4	48.3
" 7	2 "	80 cc. m/2	0.3	20.2	2.3*
" 8	23½ "	60 cc. m/2	0.9	14.3	0.4*
" 11	18 "	70 cc. m/2	0.2	87.5	24.0
" 11	21 "	80 cc. m/2	0.0	37.0	25.5
" 12	20 "	250 cc. m/6	29.6†	70.0	11.0‡
Totals			50.1	293.7	173.9

* Rabbit died during experiment.
† Average of four controls.
‡ Rabbit very sick.

TABLE IV. SUMMARY OF INTRAVENOUS INJECTIONS OF SODIUM SULPHATE.

Date.	Duration of Experiment.	Dose of Na_2SO_4.	Weight of Feces in Grams.		
			Control.	Dose by Mouth.	Dose Intravenous
Jan. 13	5 hrs.	520 cc. m/6	0.2†		9.8*
" 14	8 "	501 cc. m/6	0.0		‡15.8*
" 16	23 "	409 cc. m/6	6.2		72.8
" 18	6 "	302 cc. m/6	1.9	51.1	31.0
" 19	4 "	267 cc. m/6	4.5		3.4*
Totals			12.8		132.8

* Rabbit died.
† Average of four controls.
‡ Injection into abdomen.

show very clearly that the subcutaneous and intravenous administration of sodium sulphate increases markedly the amount of feces eliminated for some time after. When given by mouth this salt increases the amount of feces to an even greater extent.

FLUIDITY OF THE FECES.

In addition to the increase in the amount of feces, the injection of sodium sulphate also increases the proportion of water contained in them. This proportion was not measured quantitatively, so the data are not so satisfactory. In three of the experiments with subcutaneous injections the injected animals passed pasty or semifluid feces, while none of the controls did so.

With intravenous injection, however, the effect of the salt on the fluidity of the feces was most clearly shown. In these experiments the rabbits remained tied down on the board for the whole of the 4 to 8 hours during which the salt was given. They were covered with cotton batting to keep their temperature up. A warmed $\frac{M}{6}$ solution of sodium sulphate was allowed to flow from a burette continuously into the marginal vein of the ear at the rate of from 30 to 50 cc. per hour. In these experiments there was a regular progressive increase in the fluidity of the feces. At first there were well formed pellets, hard and dry. After from four to six hours these pellets began to be more moist. At first they were hard and well formed, but moister than usual on the surface, as if the moisture had entered the intestine after the pellets had been formed. Later the pellets became moist clear through, and soft. Later still they were pasty and semifluid, the masses not well formed at all, and so soft that after they had been collected the masses could hardly be distinguished. Finally, in some cases, just as the first really fluid feces were being passed the animal died. In these cases the post mortem showed that if the intestines had continued to act as they had been doing for a little longer fluid feces would have been obtained. For in these cases, the lower part of the large intestine, which usually contains hard well-formed pellets, was filled for distances of from 5 to 20 centimeters with perfectly unformed and fluid feces. In all the experiments the controls never once showed such a regular increase in the fluidity of the feces. These facts show very conclusively that the *intravenous injection of sodium sulphate increases markedly the amount of water in the feces.*

COMPARISON WITH ADMINISTRATION BY MOUTH.

When now we come to compare the effect of giving sodium sulphate by mouth with the results just described, a decided differ-

ence is observed. Fluid feces can easily and almost invariably be obtained by giving sodium sulphate by mouth. From 60 to 110 cc. of $\frac{M}{2}$ sodium sulphate or 250 to 300 cc. of the $\frac{M}{6}$ solution caused an elimination of fluid feces in every case except one, and in no case had any injurious effect on the rabbit. Why this difference? In answering this question it is essential that the *normal anatomy and physiology of the rabbit's digestive tract should be kept in mind.* The food is ordinarily very bulky, containing much cellulose, and the digestive tract is adapted to these conditions. The stomach is large and is never empty; and between the small and the large intestine is the enormous caecum, which is always filled with rather fluid, partially digested material. The food passes very slowly through the digestive tract. Thus, when I fed carrots, the feces became reddish in color, but this color was not decided until the carrots had been fed for several days and persisted for several days after the diet had been changed. When a control animal is killed the greater part of the large intestine is usually found to contain well-formed pellets, and it is only within the caecum or its immediate vicinity, that any considerable quantity of fluid feces is seen. When sodium sulphate is given by mouth the same regular increase in fluidity described for intravenous injections is obtained, and it takes about 6 hours before fluid feces are obtained. In view of these facts, then, it is obvious that the fluid feces have not been produced so much by the transformation of solid feces in the large intestine, as by the rapid conveyance of fluid feces from the caecum to the exterior. Accordingly unusual activity of the large intestine, and particularly of the caecum, would appear to be the *sine qua non* for the elimination of fluid feces. As a matter of fact when fluid feces were obtained, the peristalsis of the caecum was sometimes so violent that it could easily be seen through the muscles, skin, and hair of the abdomen.

We may now return to the explanation of why the sodium sulphate, given by mouth, is so much more effective in producing fluid feces. The reasons for this are mainly three:

1. When given by mouth the salt solution is not rapidly absorbed into the circulation, but is passed down into the caecum. Thus the solution distends this organ, and dilutes its contents, thus tending by its water and bulk alone to make the feces more fluid, and to produce peristalsis of the caecum.

2. When injected subcutaneously the solution is not rapidly absorbed into the circulation, and so does not reach the intestine in as great concentration as when given by mouth.

3. When injected intravenously it is diluted by the blood and acts all over the body, so that only a small portion reaches the intestine. But more important than this is the fact that it acts as a diuretic, and is rapidly eliminated by the kidneys.

The evidence for these three reasons will be presented in order:

1. Post mortems made six or seven hours after the solution has been given by mouth show the caecum very much distended with unusually fluid feces. This fluid must have come directly from that introduced into the stomach; for if it had been absorbed and secreted again it must have been in circulation for a while, and in that case it would have caused diuresis, which did not take place. That the mere volume of the solution has had considerable effect is shown by the fact that 350 cc. of tap water given by mouth caused the elimination of pasty and semifluid feces.[1]

2. That absorption is slow when sodium sulphate is injected subcutaneously is shown by the persistence for several days of the jelly-like oedematous mass that forms about the region of the injection. An interesting behavior of this mass was noted when $\frac{M}{2}$ sodium sulphate was injected into the dorsal region. When this was done the mass would form as usual, and then, apparently acted on by gravity, would slowly travel down the sides of the rabbit until it came to rest in the midventral line where it would persist for several days. This migration usually took 4 or 5 hours to complete. Post mortems showed a slight inflammation in the region of the swelling.

3. The rapid excretion of intravenously injected sodium sulphate is easily shown. The rabbits thus treated were constantly urinating, while the controls, and rabbits receiving the salt by mouth urinated but rarely. In one experiment, in spite of con-

[1] The most striking result produced by the water was the phenomenon, already noted by Claude Bernard, that pronounced trembling and shaking and muscular twitching was produced. This shaking was much more pronounced than similar phenomena obtained by the subcutaneous injection of sodium citrate, and was not obtained at all with larger doses of sodium citrate and sodium sulphate given by mouth.

siderable loss of urine by evaporation and in other ways, 150 cc. of the 267 cc. injected were recovered in the urine. That this urine carried with it much of the sodium sulphate introduced is self-evident, but direct evidence for it was also afforded by the oedematous jelly-like masses in the vicinity of the kidneys, and by the inflammation of kidneys and bladder.

This fact of the exceptional effectiveness of sodium sulphate when given by mouth and the explanations of the same all go to show what was already well known to us by MacCallum's investigations, that the mechanism for the production of purgation is located in the walls of the digestive tract.

EFFECTS OF BARIUM CHLORIDE.

This salt, as has been pointed out by MacCallum in a number of papers, is the most energetic of the saline cathartics. He says:[1] "For anyone to convince himself that a salt may act as a purgative when injected subcutaneously or intravenously it is only necessary to introduce a small amount of $BaCl_2$ into the blood or under the skin of a rabbit. The evacuation of large quantities of semifluid feces and the violent intestinal movements leave no room for doubt as to the action of the salt. The fact that the intravenous and subcutaneous administration of this salt as a purgative by veterinarians is in general use should be sufficient proof." Auer did not test the purgative effect of barium chloride. I have made a few experiments with this salt in order to compare the effects of *per os* and *subcutaneous* administration of a powerful saline purgative.

It has been shown that with the mild purgative sodium sulphate acts better by mouth, because the fluid is not rapidly absorbed from the digestive tract, and so has time to accumulate in the caecum, which it distends, and the contents of which it dilutes. It acts less strongly when given intravenously because it is rapidly excreted by the kidneys. Thus in both cases the sodium sulphate reaches the purgative mechanism in the intestines, but on account of the slow absorption from the intestines, it remains in contact with this mechanism longer when given by

[1] *On the Mechanism of the Physiological Action of the Cathartics. Univ. of Cal. Publications:* Berkeley, The University Press, pp. 20–21, 1906.

mouth than when given intravenously; and since this is a mild purgative a long contact is essential. With barium chloride, however, the conditions are quite different. Purgation can be obtained with such small doses that the bulk of the fluid introduced into the digestive tract cannot be of consequence in aiding purgation. Furthermore, it is very probable that it is not rapidly eliminated by the kidneys, for MacCallum has shown[1] that intravenous doses of 1 cc. of $\frac{M}{8}$ barium chloride stop the secretion of the urine. For these reasons, then, it might be expected that barium chloride would be more effective when given subcutaneously than when given by mouth, and such I have actually found to be the case.

Subcutaneous doses of 2 cc. of $\frac{M}{6}$ barium chloride never failed to cause the elimination of fluid feces within two hours. The action was very regular. Feces began to be passed within a few minutes after the injection. At first the pellets were hard and dry, but after about 30 to 40 minutes they were still hard but were covered with a good deal of surface moisture. The amount of this moisture increased, and the pellets became softer, until finally the fecal masses were entirely unformed. They were rather lumpy, some parts being pasty, while others were so fluid that they flowed through wire netting with millimeter meshes, and wet the hair about the anus. These feces were much more fluid than those of any rabbits that I have seen that have not had purgatives, so that no matter what definition of purgation be adopted it must be concluded that barium chloride does purge when given subcutaneously.

When given by mouth the action of barium chloride takes longer in manifesting itself, and is much less constant. In two of the four experiments no fluid feces were obtained, the pellets remaining distinct. In one experiment the feces were entirely unformed and mushy, but were not fluid enough to run through the millimeter meshes. In only one case were the feces really fluid, but in this case they were more fluid than any which were passed after the subcutaneous administration of barium chloride. All the rabbits receiving barium chloride subcutaneously died either during the experiment, or an hour or two after; while only

[1] *Journ. of Exp. Zoöl.*, i, p. 186, 1904.

one of the rabbits that were treated by mouth died, and that one only after many hours. The quantitative data of the experiments are contained in the following summary.

TABLE V. SUMMARY OF EXPERIMENTS WITH BARIUM CHLORIDE.

Date.	Duration of Experiment.	Dose of $\frac{M}{6}$ $BaCl_2$	Weight of Feces in Grams.		
			Control.	Dose by Mouth.	Dose subcut.
Jan. 4	2 hrs.	4 cc. divided	0.0	88.2	45.8
Feb. 7	1 hr. 25 min.	2 c .	9.2	13.5	30.3
" 25	3 hrs. 15 min.	2 cc.	0.5	28.9	29.1
" 26	1 hr. 35 min.	2 cc.	0.5	21.5	42.6
Totals			10.2	152.1	147.8

DISCUSSION OF RESULTS.

It has been shown, as may be seen in the following summary, that the subcutaneous or intravenous injection of sodium citrate, sodium sulphate, and barium chloride brings about a passage of feces from 1.3 to 23 times the amount passed by the controls.

SUMMARY OF THE TOTALS.

Purgative.	Usual Dose.	Food.	Usual Duration of Experiment.	Grams of Feces Eliminated by Experimental Animals for each Gram Passed by Controls.		
				Control.	Dose by Mouth.	Dose subcut. or Intraven.
Sodium citrate	30 cc. m/6	carrots.	4 hrs.	1		23.0
Sodium citrate	30 cc. m/6	hay, etc.	4 hrs.	1	1.6	1.3
Na_2SO_4	80 cc. m/2	mixed.	20 hrs.	1	5.9	3.5
Na_2SO_4	400 cc. m/6	"	6 hrs.	1	3.9	10.4
$BaCl_2$	2 cc. m/6	"	2 hrs.	1	14.9	14.5

When given in large doses, the subcutaneous or intravenous administration of all these salts also causes the passage of *more fluid* feces than those passed by the controls. These results confirm in every respect the conclusions arrived at by MacCallum. Our conception of purgation, then, is that the mechanism for this process is located in the wall of the digestive tract. This mechanism consists of both muscles and glands the stimulation

of which produces peristalsis and secretion. This stimulus may be supplied, among other things, by the purgative salts. One method of demonstrating this stimulation which MacCallum has described, is to open the abdomen and observe the peristalsis after the injection of the purgative salts. Auer has confirmed these observations of MacCallum, except for magnesium sulphate. But as he has been unable to demonstrate any increase in the feces eliminated after injection of these salts, Auer concludes "Peristalsis and purgation are not synonymous terms; increased peristalsis may occur during constipation." According to the conception of purgation here outlined the increased peristalsis shows that at least part of the purgative mechanism has been stimulated. But in order that this stimulus may result in the increased elimination of feces it must be kept up for a considerable time. Consequently it is easier to demonstrate increased peristalsis than increased elimination of feces. I have found that fluid feces will be produced by barium chloride after it has been in contact with the purgative mechanism for an hour and a half. Sodium sulphate, which is a much weaker purgative, requires, under the most favorable circumstances, not only 100 or 200 times as strong a dose, but *six* hours in which to produce fluid feces. This shows us that *time* is an essential factor in the process. Thus, in order to be effective in the elimination of feces the salts must not only be brought in contact with the purgative mechanism, but *they must be kept there for a certain time.* This principle should always be kept in mind in considering the different effects produced by the different methods of administering saline purgatives.

Having discussed the reasons for the difference in the results of MacCallum and Auer, I might close here were it not for the emphasis that Meltzer and Auer have laid upon MacCallum's statement that magnesium sulphate acts as a purgative when given subcutaneously.

ACTION OF MAGNESIUM SULPHATE.

In a series of papers on the action of magnesium salts Meltzer and Auer have found that in all cases these salts have a narcotic or inhibitory action, and never a stimulating effect. On the

other hand they say that MacCallum's results with saline purgatives, including magnesium sulphate, lead to the conclusion that this salt does have a stimulating effect. Speaking of MacCallum's work Meltzer and Auer[1] say: "Here we have, then, a pronounced theory supported by experiments that purgatives, magnesium sulphate included, produce peristalsis by increasing the sensitiveness of nerve and muscle of intestines; in other words, here we seem to have an instance in which magnesium salts do not inhibit but stimulate activity." Now in this quotation we have one example of a misconception that runs all through their paper. They everywhere seem to consider magnesium sulphate and magnesium salts as synonymous terms; and seem to think that MacCallum considered the purgative action of magnesium sulphate due to the magnesium which it contains. Nothing could be farther from the truth. MacCallum was very familiar with the way in which magnesium inhibits intestinal peristalsis. In fact he was the discover of this inhibition, and has mentioned it in a number of papers.[2] Concerning the purgative action of magnesium sulphate he says in his last paper,[3] concluded but a short time before his death: "This salt of course acts as a purgative because it is a sulphate and not on account of the presence of magnesium. As shown later on, magnesium chloride has an effect quite opposite to this."

Meltzer and Auer do not mention any of MacCallum's results on the inhibitory effect of magnesium in their paper on the relation of magnesium salts to peristalsis, in spite of the fact that these results are exactly in accord with their main contention that the effect of these salts is always an inhibitory or narcotic one. On the contrary, starting with MacCallum's statement that magnesium sulphate acts as a purgative when given subcutaneously, they lead one to believe that MacCallum thought that in this case magnesium stimulates peristalsis.

[1] *Amer. Journ. of Physiol.*, xvii, p. 314, 1906.

[2] MacCallum: *Amer. Journ. of Physiol.*, x, p. 259, 1904; also, *Arch. f. d. ges. Physiol.*, civ, p. 425, 1904; *Journ. of Exp. Zoöl.*, i, p. 179, 1904; *Univ. of Cal. Publications*, Physiology, ii, p. 47, 1905; this *Journal*, i, p. 339, 1906; *On the Mechanism of the Physiological Action of the Cathartics. Univ. of Cal. Publications:* Berkeley, The University Press, p. 38, 1906.

[3] *On the Mechanism of the Physiological Action of the Cathartics. Univ. of Cal. Publications:* Berkeley, The University Press, p. 21, 1906.

As MacCallum has often stated, the theoretical ideas upon which his conception of purgation was based are due to Loeb.[1] In a series of papers, the first of which was published in 1899, Loeb[2] found that in producing muscular twitchings and some other phenomena sodium and barium salts were particularly effective, and were counteracted by calcium and magnesium salts. On account of the counteracting effect of calcium the sodium salts that were most effective were those that precipitate calcium. For this reason sodium sulphate is more effective than sodium chloride, because it not only adds sodium but takes away calcium from the solution. Similarly, magnesium sulphate will remove calcium while it adds magnesium. Now since calcium inhibits more strongly than magnesium, the addition of magnesium sulphate should have something of a stimulating effect, not because of the magnesium but because of the SO_4-ion which precipitates the calcium. In this way, then, the purgative effect of magnesium sulphate is explained; and it can be seen at once that, according to this view, magnesium sulphate should be considered a rather unsatisfactory purgative, as it contains both stimulating and inhibitory components.

It is of course a fundamental precaution in interpreting the effect of any salt, to consider the possibility of either ion having produced the effect, and Meltzer and Auer have on other occasions been careful in taking this precaution. In the paper under discussion, however, they have on several occasions failed to do so. Thus, when testing the effect of magnesium salts in stopping the peristalsis caused by barium chloride they find in one experiment that magnesium sulphate stops the peristalsis, and conclude from this that magnesium salts do inhibit the peristalsis. Now while this conclusion is right it is not justified by the fact, for it is well known that magnesium sulphate reacting with barium chloride will produce barium sulphate which is insoluble. The barium would thus be removed from solution and could no longer produce its characteristic effect. The injection of magnesium chloride, on the other hand, which was also tried by the authors, does show that the magnesium inhibits the peristalsis.

[1] *Univ. of Chicago Decennial Publications*, x, p. 10, 1902.
[2] *Festschrift für Fick*, Braunschweig, p. 99, 1899.

In conclusion, then, it may be stated that a re-examination of the effect of intravenous and subcutaneous injection of sodium citrate, sodium sulphate, and barium choride has shown that these salts markedly increase the amount of feces eliminated, and also, when given in larger doses, markedly increase the fluidity of the feces. These results confirm in every respect the conclusions of MacCallum. The reasons why Auer, and Meltzer and Auer have been unable to confirm this result seem to be: (1) they used only the milder purgatives mentioned by MacCallum, and (2) in using these they did not keep adequate controls and thus failed to notice the increase in the amount of feces which they were probably getting.

SUMMARY.

1. In any experiments dealing with the quantity and character of the feces eliminated it is absolutely essential that the feces of the experimental animals should be compared with those of control animals kept at the same time and treated in the same way as the experimental animals, except of course that no salts are given to them. The neglect of this precaution is largely responsible for the different results arrived at by Auer and MacCallum.

2. When small doses of mild purgatives are given a bulky diet will sometimes largely mask any effect that might be due to the purgative. In such experiments, therefore, a more concentrated diet should be given.

3. The subcutaneous injection of small doses of sodium citrate was found to increase the feces eliminated for the next 3 to 5 hours to 23 times the amount eliminated by the controls.

4. The intravenous injection of maximal doses of sodium sulphate was found to increase the feces 10 times.

5. The subcutaneous injection of 2 cc. of barium chloride was found to increase the feces eliminated for the next two hours 14½ times.

6. Large subcutaneous or intravenous doses of all three of these salts make the feces eliminated unusually moist. After barium chloride the feces even become partially fluid.

7. Fluid feces can be more easily obtained with sodium citrate when it is given by mouth because it usually takes more than

100 cc. of an $\frac{M}{6}$ solution to produce fluid feces, and this amount is almost always fatal when given subcutaneously.

8. With sodium sulphate fluid feces are more easily obtained when the salt is given by mouth because:

a. It is but slowly absorbed from the digestive tract, and so is passed on down into the caecum which it distends and the contents of which it dilutes. It thus remains in contact with the walls of the digestive tract for a long time and has a chance to affect the mechanisms for peristalsis and secretion which are contained in the walls.

b. It is but slowly absorbed from the subcutaneous tissue, and so never reaches the walls of the digestive tract in sufficient concentration to produce its maximum effect.

c. When introduced into the circulation it acts as a diuretic and is thus rapidly eliminated and never reaches the intestines in sufficient concentration and for a long enough time to produce its maximum effect before it has killed the rabbit.

9. With barium chloride fluid feces are more easily obtained when the salt is given subcutaneously than when it is given by mouth.

10. In considering the effect of any salt it should always be borne in mind that the effect may be due to the activity of either anion or cation. Magnesium sulphate acts as a purgative on account of its anion.

11. All of these results go to show that MacCallum's conclusions are correct: The mechanism for causing purgation is in the intestinal walls and is stimulated whenever any saline purgative reaches it in sufficient concentration no matter in what way that purgative may have been introduced into the body.

THE PROTEINS OF THE PEA (*Pisum Sativum*).[1]

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According to former investigations made in this laboratory the seeds of the garden pea (*Pisum Sativum*) contain three different proteins: Legumin, vicilin and legumelin. The two former are globulins of similar composition and properties which were separated from one another by fractional precipitation from sodium chloride solutions, the vicilin being more soluble than the legumin in dilute saline solutions while the legumelin remained in solution after dialysis and was separated by heating the solution to 80°. The most marked difference between legumin and vicilin was shown by the behavior of their solutions on heating. Sodium chloride solutions of legumin remain perfectly clear when heated to 100°, those of vicilin become turbid at 90°, at 95° a flocculent coagulum separates and when heated at 100° for some time the vicilin is almost completely coagulated. Legumin contains a little less carbon and a little more nitrogen than vicilin and distinctly more sulphur. Vicilin contains less sulphur than any protein thus far isolated. By repeated precipitation the amount of sulphur was found to diminish from 0.23 per cent to 0.08 per cent.[2]

The name legumin has long been used to designate various protein preparations obtained from many of the leguminous seeds. Most of these preparations were formerly obtained by extraction with alkali and precipitation with dilute acid and therefore represented products of doubtful character. Later investigations showed that most of these proteins were globulins which could be

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] Cf. Osborne and Campbell, *Journ. Amer. Chem. Soc.*, xx, p. 410, 1898.

extracted by sodium chloride solution and that several of the preparations previously called legumin were certainly different substances. The investigations of many different leguminous seeds made in this laboratory have shown that from the pea, *Pisum Sativum*, horse bean, *Vicia Faba*, lentil, *Ervum lens*, and vetch, *Vicia Sativa*, preparations of the globulin can be obtained which agree strictly in properties and composition with one another but are distinctly different from those obtained from seeds of the genus *Phaseolus* and other kinds of legumes. The writer has therefore designated this protein as legumin, for it undoubtedly represents the substance to which earlier investigators most frequently intended to apply this name. In the seeds of the pea, horse bean and lentil the legumin is associated with another protein which has been called vicilin by the writer. The history of legumin and the characteristics of the proteins of the seeds above mentioned has been discussed in papers from this laboratory.[1]

Legumelin is an albumin-like protein which is not precipitated by dialysis and is coagulated by heating its solutions to about 80°. The greater part of the legumelin separates between 60° and 65° but its complete coagulation is not effected below 80°.

The composition of legumelin is distinctly different from that of legumin or vicilin. In properties and composition it closely resembles leucosin found in the embryo of wheat and it is probably a tissue protein rather than a reserve food protein of the endosperm. Proteins of the same composition and properties as the legumelin of the pea have been found in a large number of other leguminous seeds, *e.g.*, lentil, horse bean, vetch, adzuki bean, cow pea and soy bean.

As a complete separation of legumin from vicilin by fractional precipitation from sodium chloride solutions can be obtained only by the sacrifice of a large part of the mixed globulins and the expenditure of much time and labor we have studied the results obtained by fractional precipitation with ammonium sulphate and found that this method yields products of the same properties and ultimate composition as those previously obtained from

[1] Cf. Osborne and Campbell: *Journ. Amer. Chem. Soc.*, xviii, p. 583, 1896. *Ibid.*, xx, pp. 348, 362, 393, 406 and 410, 1898.

sodium chloride solutions. By this method it is possible to prepare large quantities of these proteins without much loss of material and with comparative ease.

The results given by this method are shown by the following experiment:

The pea-meal was extracted with 10 per cent sodium chloride solution and the extract, filtered clear, was saturated with ammonium sulphate. The precipitate thus produced was dissolved in dilute ammonium sulphate solution and the resulting solution dialyzed for five days. The precipitated globulin was dissolved in sodium chloride solution and again precipitated by saturation with ammonium sulphate, the precipitate dissolved in sodium chloride solution and, after filtering perfectly clear, the solution was dialyzed for ten days. The dialysis precipitate was then suspended in 1000 cc. of water and dissolved by adding 76 grams of ammonium sulphate. By adding 380 grams more ammonium sulphate, thereby raising the concentration to six-tenths saturation, a considerable precipitate was produced. This was filtered out, washed with six-tenths saturated sulphate solution, dissolved in dilute sulphate solution and the resulting clear solution dialyzed for seven days. The globulin that precipitated was completely soluble in 10 per cent sodium chloride solution and gave no coagulum when this solution was boiled.

The remainder of the globulin, when washed with water and alcohol and air-dried, weighed 15.3 grams and, dried at 110°, had the following composition: C, 51.74; H, 7.14; N, 17.77 per cent.

The filtrate from the precipitate produced by six-tenths saturation was raised to seven-tenths saturation but, as only a trace of precipitate formed, the saturation was raised to eight-tenths and the resulting precipitate filtered out. This was dissolved in dilute ammonium sulphate solution and the clear solution was dialyzed for seven days. The precipitate that formed, when filtered out, washed and air-dried, weighed 7.35 grams. It was completely soluble in dilute sodium chloride solution and largely coagulated when this solution was heated in a boiling water-bath.

Dried at 110° this preparation had the following composition: C, 52.25; H, 7.28; N, 17.17 per cent. The solution filtered from the precipitate at eight-tenths saturation, when completely saturated with ammonium sulphate, yielded a small precipitate which

when redissolved and precipitated by dialysis, gave 0.69 gram of substance which was wholly soluble in dilute sodium chloride solution, coagulated by heating to 100° and when dried at 110° had the following composition: C, 52.17; H, lost; N, 17.08 per cent. A repetition of this experiment, making the separation at seven-tenths saturation, gave essentially the same results, namely, 26.5 grams of legumin, containing C, 51.89; H, 6.83: N, 17.78 per cent, and 9.5 grams of vicilin, containing C, 52.35; H, 7.15; N, 16.90 per cent.

A third extraction gave at five-tenths saturation 17.76 grams of globulin containing 17.77 per cent of nitrogen; between five-tenths and six-tenths 9.62 grams containing 17.99 per cent of nitrogen; between seven-tenths and eight-tenths 10.03 grams containing 17.18 per cent nitrogen, and between eight-tenths and complete saturation 7.46 grams, containing 17.00 per cent of nitrogen.

It is thus evident that separation by fractional precipitation with ammonium sulphate yields products of the same composition and properties as those formerly obtained by fractional precipitation from sodium chloride solutions and as this separation is effected without difficulty and with little loss of substance, it affords an excellent method for preparing large quantities of these two proteins in as pure a state as it is possible to obtain them in any way known to us.

We accordingly prepared large quantities of these proteins for hydrolysis by extracting the pea-meal with 10 per cent sodium chloride solution, filtering the extract perfectly clear and dialyzing until nearly all of the chlorides were removed. The dialysis precipitate was then dissolved in one-tenth saturated ammonium sulphate solution, the resulting solution filtered clear, and enough ammonium sulphate crystals dissolved in it to bring it to six-tenths saturation.

The precipitate thus produced was dissolved in sodium chloride solution and, after filtering perfectly clear, the solution was dialyzed until free from chloride. The resulting precipitate of legumin was then washed with water, dilute and absolute alcohol and dried over sulphuric acid.

The filtrate from the precipitate produced by six-tenths saturation with ammonium sulphate was completely saturated with

this salt, the precipitated protein dissolved in sodium chloride solution and, after filtering perfectly clear, the solution was dialyzed until free from chlorides. After washing the dialysis precipitate with water and alcohol it was dried over sulphuric acid. The product thus obtained formed our preparation of vicilin. The filtrate from the precipitate produced by the first dialysis of the original sodium chloride extract of the pea-meal was heated to 80° in a water-bath, the voluminous coagulum was washed with water and dehydrated with absolute alcohol, giving a preparation of legumelin. By this method 1840 grams of legumin, 865 grams of vicilin and 790 grams of legumelin were obtained.

This legumin contained 17.75 per cent of nitrogen (Kjeldahl), 0.46 per cent of sulphur and 0.48 per cent of ash, and when dissolved in sodium chloride solution was not coagulated by heating to 100°. The vicilin contained 17.15 per cent of nitrogen, 0.26 per cent of sulphur and 0.41 per cent of ash. Dissolved in sodium chloride solution it was abundantly coagulated on heating to 100°.

These preparations of legumin and vicilin agreed in composition and deportment on heating with those obtained by fractional precipitation from sodium chloride solution and undoubtedly represent the same fractions of the total globulin of this seed as were formerly described under these names.

HYDROLYSIS OF LEGUMIN FROM THE PEA.[1]

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(*From the Laboratory of the Connecticut Agricultural Experiment Station.*)

(Received for publication, May 9, 1907.)

The legumin used for this hydrolysis was prepared in the way described in the preceding paper.

Five hundred grams, equal to 449 grams of ash- and moisture-free legumin, were dissolved by warming in the water-bath with a mixture of 500 cc. of water and 500 cc. of hydrochloric acid of specific gravity 1.2. The solution was then boiled in a bath of oil for 15 hours.

The hydrolysis solution was then concentrated under reduced pressure to two-thirds of the original volume, saturated with hydrochloric acid gas and allowed to stand at 0°.

The glutaminic acid hydrochloride, freed from ammonia by boiling with baryta and recrystallized from strong hydrochloric acid, weighed 77.34 grams, which is equivalent to 61.95 grams of glutaminic acid. The free acid decomposed at 202°–203° with effervescence to a clear oil and gave the following analysis:

Carbon and hydrogen: 0.2272 gm. substance gave 0.3380 gm. CO_2 and 0.1283 gm. H_2O.

Calculated for $C_5H_9O_4N$: C = 40.81; H = 6.12 per cent.

Found.....C = 40.57; H = 6.27 " "

The filtrate from the hydrochloride of glutaminic acid was concentrated under reduced pressure to a thick syrup and esterified with alcohol and dry hydrochloric acid gas in the usual manner. The aqueous layer remaining after extracting the esters with ether, was then freed from inorganic salts and the esterification

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

repeated. As considerable ester, soluble in ether, was obtained, the whole operation was again repeated, but this third treatment yielded only an insignificant quantity of ether-soluble ester.

The ether was then distilled off on the water-bath and the residue fractioned under diminished pressure with the following result:

Fraction.	Temp. of bath up to	Pressure.	Weight.
I	55°	10 mm.	33.82 gm.
II	85°	10 "	29.88 "
III A	100°	10 "	27.06 "
III B		0.75 "	49.10 "
IV A	140°	0.75 "	65.08 "
IV B	200°	0.65 "	37.40 "
		Total,	242.34 "

The undistilled residue weighed 107 grams.

Fraction I. (Temperature of bath up to 55°; pressure, 10 mm.; weight, 33.82 grams.) This fraction, which consisted largely of alcohol and ether, was evaporated to dryness with strong hydrochloric acid and the residue esterified with alcohol and dry hydrochloric acid gas. The glycocoll ester hydrochloride that separated on standing at 0°, weighed 0.56 gram. The filtrate was worked up conjointly with the glycocoll filtrate of Fraction II.

Fraction II. (Temperature of bath, up to 85°; pressure, 10 mm.; weight, 29.88 grams.) The ester of this fraction was saponified by boiling with water until the alkaline reaction had ceased. The dried amino-acids were freed from prolin with boiling absolute alcohol and re-esterified with dry alcohol and hydrochloride acid gas. The weight of glycocoll ester hydrochloride that separated at 0°, was 2.66 grams, equivalent to 1.43 gram of glycocoll. It crystallized from alcohol in the characteristic needles melting at 144°.

Chlorine: 0.3054 gm. substance gave 0.3174 gm. AgCl.
Nitrogen: 0.4008 gm. substance required 4.05 cc. $\frac{5N}{7}$ HCl.
Calculated for $C_4H_{10}O_2NCl$: Cl = 25.45; N = 10.04 per cent.
Found..... Cl = 25.70; N = 10.11 " "

In the filtrate from the glycocoll the free amino-acids were regenerated and submitted to fractional crystallization. The less

soluble fractions yielded 4.23 grams of leucin, decomposing at 298°.

Carbon and hydrogen: 0.1077 gm. substance gave 0.2166 gm. CO_2 and 0.0960 gm. H_2O.

Calculated for $C_6H_{13}O_2N$: C = 54.96; H = 9.92 per cent.
Found.....C = 54.85; H = 9.90 " "

The remainder of the fraction consisted mainly of alanin. Valin, if present at all, was in such small quantity that its isolation was not attempted.

The alanin weighed 9.34 grams.

Carbon and hydrogen: 0.1477 gm. substance gave 0.2184 gm. CO_2 and 0.1067 gm. H_2O.

Calculated for $C_3H_7O_2N$: C = 40.45; H = 7.86 per cent.
Found .....C = 40.33; H = 8.02 " "

The substance decomposed at about 290°.

Fraction III. (A: Temperature of bath up to 100°; pressure, 10 mm.; weight, 27.06 grams. B: Temperature of bath up to 100°; pressure, 0.75 mm.; weight, 49.10 grams.)

This fraction consisted almost entirely of the esters of leucin and prolin. After saponifying with boiling water, the solutions were evaporated to dryness under reduced pressure and the prolin extracted with boiling alcohol. The part remaining undissolved yielded on fractional crystallization 31.65 grams of leucin.

Carbon and hydrogen: 0.1855 gm. substance gave 0.3742 gm. CO_2 and 0.1670 gm. H_2O.

Calculated for $C_6H_{13}O_2N$: C = 54.96; H = 9.92 per cent.
Found .....C = 55.02; H = 10.00 " "

The substance decomposed at 298°.

The alcohol extracts containing the prolin of Fractions II and III were worked up conjointly. After separating from a small quantity of alcohol-insoluble substance by repeated evaporations to dryness under reduced pressure, the substance was converted into the copper salt and the laevo separated from the racemic prolin copper salt with boiling absolute alcohol.

The insoluble racemic salt was freed from copper with hydrogen sulphide and the filtrate from copper sulphide evaporated to dryness under reduced pressure. The residue proved entirely soluble in absolute alcohol. For identification the racemic prolin was

again converted to the copper salt and the latter recrystallized from water. The yield of the air-dried copper salt was 2.85 grams equivalent to 2.00 grams of prolin.

Water: 0.1278 gm. substance, dried in air, lost 0.0138 gm. H_2O at 110°.
Copper: 0.1134 gm. substance, dried at 110°, gave 0.0305 gm. CuO.
Calculated for $C_{10}H_{16}O_4N_2Cu.2H_2O$: $H_2O = 10.99$ per cent.
Found..... $H_2O = 10.80$ " "
Calculated for $C_{10}H_{16}O_4N_2Cu$: Cu = 21.81 per cent.
Found .....Cu = 21.49 " "

The copper salt of the laevo-prolin, soluble in alcohol, weighed, when dried at 100°, 15.80 grams, equivalent to 12.47 grams of prolin.

For identification the phenylhydantoin was employed. It crystallized from water in the characteristic flat prisms, melting at 143°.

Carbon and hydrogen: 0.2241 gm. substance gave 0.5456 gm. CO_2 and 0.1152 gm. H_2O.
Calculated for $C_{13}H_{12}O_2N_2$: C = 66.67; H = 5.57 per cent.
Found .....C = 66.40; H = 5.71 " "

Fraction IV. (A: Temperature of bath, up to 140°; pressure, 0.75 mm., weight, 65.08 grams. B: Temperature of bath, up to 200°; pressure, 0.65 mm.; weight, 37.40 grams.)

From this fraction the ester of phenylalanin was removed in the usual manner by shaking out with ether and the ether layer washed repeatedly with an equal volume of water. After saponification with strong hydrochloric acid, there were obtained 20.54 grams of the hydrochloride of phenylalanin equivalent to 16.82 grams of free phenylalanin. For identification the copper salt was employed.

Copper: 0.1422 gm. substance, dried at 110°, gave 0.0284 gm. CuO.
Calculated for $C_{18}H_{20}O_4N_2Cu$: Cu = 16.24 per cent.
Found .....Cu = 15.96 " "

The aqueous layer was saponified by warming with an excess of baryta and the racemized aspartic acid isolated as the barium salt. The yield was 13.43 grams.

Carbon and hydrogen: 0.2597 gm. substance gave 0.3451 gm. CO_2 and 0.1279 gm. H_2O.
Calculated for $C_4H_7O_4N$: C = 36.09; H = 5.26 per cent.
Found .....C = 36.24; H = 5.47 " "

In the filtrate from the barium aspartate no glutaminic acid could be isolated as the hydrochloride. The remainder of the fraction consisted essentially of aspartic acid and serin and for the isolation of the former substance the copper salt was employed.[1] The weight of air-dry copper aspartate was 21.46 grams equivalent to 10.36 grams of aspartic acid.

Copper: 0.1317 gm. substance, dried in air, gave 0.0383 gm. CuO.
Nitrogen: 0.4428 gm. substance, dried in air required 2.33 cc. $\frac{5N}{7}$ HCl.
Calculated for $C_4H_5O_4NCu.4\frac{1}{2}\ H_2O$: Cu = 23.07; N = 5.08 per cent.
Found ..... Cu = 23.23; N = 5.26 " "

The filtrate from the copper aspartate was freed from copper with hydrogen sulphide. By fractional crystallization there were obtained 2.45 grams of pure serin, which decomposed at about 240°.

Carbon and hydrogen: 0.2300 gm. substance gave 0.2909 gm. CO_2 and 0.1389 gm. H_2O.
Calculated for $C_3H_7O_3N$: C = 34.29; H = 6.67 per cent.
Found ..... C = 34.49; H = 6.71 " "

Residue after Distillation. The residue that remained after distilling off the esters weighed 107 grams. It was dissolved in hot alcohol. The solution separated on cooling a precipitate weighing 3.32 grams. The filtrate was evaporated to dryness, the residue was taken up in water and saponified by warming with an excess of baryta. After removing the baryta with sulphuric acid, the solution was concentrated and saturated with hydrochloric acid gas. After prolonged standing at 0°, no hydrochloride of glutaminic acid could be made to separate, even at different concentrations of the solution.

TYROSIN.

Fifty grams of the legumin, equal to 44.89 grams ash- and moisture-free, were hydrolyzed with three parts, by weight, of sulphuric acid and six parts of water for eleven hours and, after removing the sulphuric acid with an equivalent quantity of barium hydroxide, the solution was concentrated until a crystal-

[1]Fischer and Dörpinghaus: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 474, 1902.

line product separated. This was filtered out and the filtrate further concentrated and a second separation obtained. These were then recrystallized from hot water and the crude tyrosin which was thus obtained was dissolved in boiling water, decolorized with bone black and recrystallized. Dried at 110° this tyrosin weighed 0.7 gram, equal to 1.56 per cent.

Nitrogen: 0.3162 gm. substance required 2.40 cc. $\frac{5N}{7}$ HCl.
Calculated for $C_9H_{11}O_3N$: N = 7.73 per cent.
Found ..... N = 7.59 " "

The filtrate and washings from the tyrosin were concentrated and histidin, arginin and lysin determined by the method of Kossel and Patten.[1]

HISTIDIN.

The solution of the histidin contained nitrogen equal to 1.0870 gram of histidin, equal to 2.42 per cent.

Histidin solution = 500 cc. 50 cc. required 5.90 cc. $\frac{5N}{7}$ HCl = 0.2950 gm. N in 500 cc. = 1.087 gm. histidin or 2.41 per cent.

This histidin was identified as the dichloride. It decomposed at about 232°–233° and gave, on warming, the biuret reaction.

Chlorine: 0.1481 gm. substance gave 0.1854 gm. AgCl.
Calculated for $C_6H_{11}O_2N_3Cl_2$: Cl = 31.14 per cent.
Found ..... Cl = 30.95 " "

ARGININ.

The solution of the arginin contained nitrogen equal to 4.542 grams of arginin or 10.12 per cent.

Arginin solution = 1000 cc. 50 cc. required 7.2 cc. $\frac{5N}{7}$ HCl = 1.44 gm. N in 1000 cc. = 4.47 gm. arginin. Adding 0.072 gm. for the solubility of the arginin silver = 4.542 gm. or 10.12 per cent.

The arginin was identified as the copper nitrate double salt.

Water: 0.1937 gm. substance air dry lost 0.0189 gm. H_2O at 110°.
Copper: 0.1019 gm. substance, dried at 110°, gave 0.0152 gm. CuO.
Calculated for $C_{12}H_{28}O_4N_8Cu\ (NO_3)_2 . 3\ H_2O$: H_2O = 9.16 per cent.
Found ..... H_2O = 9.76 " "
Calculated for $C_{12}H_{28}O_4N_8Cu\ (NO_3)_2$: Cu = 11.87 per cent.
Found ..... Cu = 11.92 " "

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[1] Kossel and Patten: *Zeitschr. f. physiol. Chem.*, xxxviii, p. 39, 1903.

LYSIN.

The lysin was isolated and identified as picrate, of which 4.95 grams were obtained, equal to 4.29 per cent.

Nitrogen: 0.1232 gm. substance, dried at 100°, gave 20.6 cc. moist N_2 at 19.5° and 752.1 mm.

Calculated for $C_6H_{14}O_2N_2 . C_6H_3O_7N_3$: N = 18.70 per cent.

Found ..... N = 18.96 " "

CYSTIN.

Owing to the very small amount of sulphur in legumin no attempt was made to separate cystin.

The results of this hydrolysis are given in the following table:

HYDROLYSIS OF LEGUMIN FROM THE PEA.

Glycocoll	0.38 per cent.
Alanin	2.08 " "
Valin	not isolated
Leucin	8.00 per cent.
Prolin	3.22 " "
Phenylalanin	3.75 " "
Aspartic acid	5.30 " "
Glutaminic acid	13.80 " "
Serin	0.53 " "
Cystin	not determined
Tyrosin	1.55 per cent.
Arginin	10.12 " "
Lysin	4.29 " "
Histidin	2.42 " "
Ammonia	1.99 " "
Tryptophan	present
Total	57.43 " "

ON THE NUCLEIN FERMENTS OF EMBRYOS.

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(Received for publication, May 29, 1907.)

The existence in the tissues of amidases capable of causing the conversion of the amidopurins (guanin and adenin) into the oxypurins (xanthin and hypoxanthin) has been repeatedly demonstrated.[1] Since one of the amidopurins (adenin) was found transformed by the spleen while the other under precisely the same conditions was not, it became necessary to assume that two ferments are concerned in those glands which can effect both decompositions.[2] This exceptional behavior of the spleen, however, was afterwards found to characterize the animal species (pig) as other spleens (ox) can cause the decomposition of both amidopurins.[3] Having noted the different distribution of the ferments in the spleens, we undertook an investigation to find whether this difference is confined to the spleen or is extended to other organs, and we included in our study the ferment xanthooxidase.[4] We observed most remarkable differences of ferment distribution in the livers of four animal species, ox, pig, dog and rabbit. The matter is clearly set forth in the following diagrams, in which solid lines represent the undoubted existence of the ferment and dotted lines indicate that the ferment is totally absent, exists in traces or can only occasionally be found.[5] The first diagram will explain the other four.

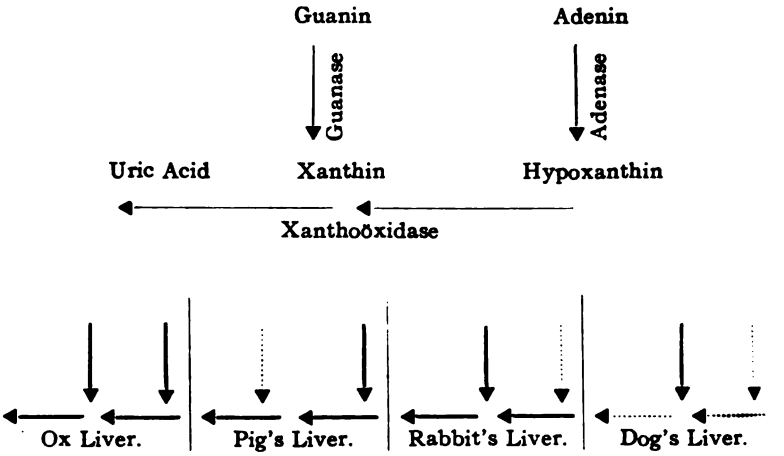
[1] Jones: *Zeitschr. f. physiol. Chem.*, xli, p. 101, 1904; xlii, p. 35, 1904. Jones and Partridge: *Ibid.*, xlii, p. 343, 1904.

[2] Jones and Winternitz: *Ibid.*, xliv, p. 1, 1905.

[3] Jones: *Ibid.*, xlv, p. 84, 1905.

[4] Spitzer: *Arch. f. Physiol.*, lxvi, p. 192, 1899. Wiener: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 373.

[5] Jones and Austrian: *Zeitschr. f. physiol. Chem.*, xlviii, p. 110, 1906.



This striking difference of nuclein ferments in the livers of different animal species at once suggests that the adult gland will be found different from the embryo gland in regard to its ferments. In order to test this hypothesis we selected the pig's liver. As the adult gland contains two of the ferments while the third is absent, we would be in a position to observe differences whether the embryo gland should take on enzymic functions as development proceeds or lose those already acquired. Our results show conclusively that the former is the case. In the earlier stages of embryonic development the pig's liver contains none of the nuclein ferments. As development proceeds, adenase makes its appearance while xanthoöxidase and guanase are still absent. The adult liver contains both adenase and xanthoöxidase, but not guanase. Our attention was first directed to guanase.

Experiments with liver extract of pig embryos of various lengths, from 80 to 200 millimeters, to which guanin had been added, showed in every case the absence of a ferment which can cause the transformation of guanin into xanthin, since in all these experiments the added guanin could be recovered unchanged after the digestion. The failure of guanase in these glands, however, is more forcibly shown in the experiments described below, in which adenin was added to the gland extract. In all cases a small amount of guanin was obtained, showing that there is no trace of guanase present, since the gland cannot transform the

small amount of guanin which is formed from its own nucleic acid. So far as guanase is concerned, the livers of pig embryos of various ages do not differ from the adult liver.

For the work on adenase, pig embryos of various lengths were sorted into groups which varied 5 millimeters in length. The livers which had been carefully removed and trimmed were ground with sand and a known weight of the paste was treated with five parts of chloroform water and allowed to stand in well closed vessels for 24 hours, after which the cloudy aqueous fluid was strained through linen. To the fluid thus obtained was added adenin sulphate in such an amount as would easily have been transformed by a similarly prepared extract of the adult liver. The results with embryos up to a length of 150 millimeters were uniform. The introduced adenin could be recovered as nearly quantitatively as one could reasonably expect, but no trace of a purin body resembling hypoxanthin could be detected.

We will describe one experiment. Forty-one grams of liver from embryos 145–150 millimeters in length were extracted with water as described. To 205 cc. of this fluid were added 250 milligrams of adenin sulphate and 5 cc. of chloroform. The material was digested at 47° with frequent agitation for 7 days. The product was then diluted with an equal volume of water and after the addition of a drop of acetic acid, heated to boiling. The pale yellow fluid was filtered from the coagulum, treated with 7 cc. of 25 per cent sulphuric acid, and evaporated to about 75 cc. The material was then boiled for about 10 minutes to decompose any trace of proteid present, and the purin bodies precipitated in the usual way with ammonia and silver nitrate. The silver precipitate was decomposed with hydrochloric acid and the filtrate from silver chloride evaporated to dryness. The residue dissolved easily in water leaving a negligible quantity of pigmented material which was submitted to a color test for xanthin, but with negative results. The pale yellow acid solution was decolorized with a little animal charcoal and evaporated again to dryness for the expulsion of the greater part of hydrochloric acid. The crystalline product was finally taken up in water and treated with a slight excess of picric acid, and the yellow precipitate of adenin picrate was quickly filtered off. The picrate weighed when dry 402 milligrams, and was found to melt at 281°. This is practic-

ally a quantitative recovery of the added adenin (90 per cent). The adenin picrate obtained in three such experiments was converted into the sulphate which was analysed with the following results:

0.2016 gm. required 5.64 cc. sulphuric acid (1 cc. = 0.0124 gm. N)		
0.1136 " " 3.17 cc. " " (1 cc. = 0.0124 gm. N)		
	Calculated:	Found:
N	34.51 per cent.	34.68; 34.60 per cent.

The mother liquor from adenin picrate was treated with sulphuric acid and ether for the removal of picric acid, and the xanthin bases precipitated with silver nitrate and ammonia. The very small precipitate was treated in the usual way and a few milligrams of a base finally obtained which would not dissolve in 2.5 per cent ammonia and which gave characteristic microscopic silky needles when treated on a slide with a drop of hot hydrochloric acid. Assuming that this gland contains the widely distributed ferment nuclease, the presence of a trace of guanin in this connection speaks strong for the absence of guanase. Thus the livers of pig embryos up to 150 millimeters do not contain adenase.

A similar series of experiments was made with livers of pig embryos of various lengths from 165 to 200 millimeters. These gave practically uniform results; the presence of guanase is still incapable of demonstration, but adenase has now made its appearance. We will describe one experiment.

Three hundred and five cubic centimeters of extract of liver from embryos 165–170 millimeters were treated with 250 milligrams of adenin sulphate and the material digested at 38° for 10 days. The treatment of the products (except for the previous removal of a trace of guanin) was the same as that described above to the point where adenin was precipitated with picric acid. A specimen of this material, however, did not produce even a cloud when treated with picric acid. The disappearance of the added adenin being thus proven, the bases were again precipitated with ammonia and silver nitrate and the silver precipitate decomposed with sulphuretted hydrogen. The filtrate from silver sulphide was evaporated to dryness, and the residue crystallized out of 6.4 per cent nitric acid. On cooling, the acid solution deposited

226 mg. of material which consisted uniformly of the characteristic crystals of hypoxanthin nitrate, and which failed altogether to respond to the xanthin color reaction with nitric acid and caustic soda.

The finding of hypoxanthin in this experiment not only shows the presence of adenase in the glands employed, but proves just as conclusively that xanthoöxidase has not yet appeared in any very appreciable amount, since we have satisfied ourselves that extracts of adult pig's liver under precisely the same conditions would have changed this hypoxanthin to xanthin or to a mixture of xanthin and uric acid.

We have described only two of a number of experiments made, but our results have been so uniform that we can draw a sharp conclusion. The liver of the pig embryo up to a length of 150 millimeters does not contain any of the nuclein ferments (guanase, adenase, xanthoöxidase). As the length of embryo increases from 150 to 170 millimeters adenase makes its appearance and this condition is maintained until a length of 200 millimeters is reached. At some later period, perhaps after birth, xanthooxidase appears but guanase is not found even in the adult pig's liver.

The results described in this paper may throw some light on certain differences between results which we have obtained and those which have been described by others. One of us with Partridge found among the products of the self-digestion of pig's pancreas (the species was not named) xanthin and hypoxanthin, but neither guanin nor adenin; and that ox pancreas (the species was not named) is capable of slowly transforming added guanin into xanthin. Schenck[1] later found among the products of self-digestion of the pancreas of both species, guanin and hypoxanthin, but neither xanthin nor adenin, and explained his results upon the hypothesis that guanase and adenase are different ferments. After this work on the pancreas had been repeated many times with results essentially as originally described, we were somewhat surprised upon one occasion to find a small amount of guanin among the products of the self-digestion of pig's pancreas. We

[1] *Zeitschr. f. physiol. Chem.*, xliii, p. 406.

have since obtained this result occasionally but always find in the presence of this small amount of guanin a larger amount of xanthin.

On one occasion we were able to show a trace of xanthoöxidase in pig's spleen, but in spite of an enormous number of experiments preceding and subsequent to this one we have not been able to demonstrate the ferment in this tissue.

In his attempt to prove the identity of guanase and adenase, Schittenhelm described experiments to show an appreciable transformation of guanin into xanthin. In one of these experiments, however, a perfectly enormous amount of guanin was introduced and a mere trace of xanthin found after the digestion.

Finally: we stated in our last contribution to this subject that rabbit's liver contains very little, if any, adenase. After two unsuccessful attempts (and we presume the results of these two agree with our own) Schittenhelm finally succeeds in "activating" the ferment and claims to show that this tissue can cause the conversion of adenin into hypoxanthin.[1]

Having in mind some of these differences, we concluded in our last contribution that not only do animal species differ in the distribution of these ferments, but that individuals of the same species may differ from one another. It is possible that some at least of these individual differences may be accounted for by a difference in age.

[1] Schittenhelm: *Zeitschr. f. physiol. Chem.*, 1, p. 30.

PROCEEDINGS OF THE

AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS,

IN SESSION IN

Washington, D. C., May 8 and 9, 1907.

EDITED BY THE SECRETARY.

Summary of Meetings.

1. *Wednesday morning, May 8*, at the George Washington Medical College.

2. *Wednesday afternoon, May 8*, at the George Washington Medical College.

3. *Thursday morning, May 9*, at the George Washington Medical College. Joint session with the American Physiological Society.

4. *Thursday evening, May 9*, at the Cosmos Club. Joint session with the Washington Section of the American Chemical Society.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

Washington, D. C., May 8 and 9, 1907.

First Meeting.

George Washington Medical College. Wednesday morning, May 8.

Presiding officer: The Vice-President, John J. Abel.

ON THE BEHAVIOR OF FROG'S MUSCLE TOWARD ACIDS.

By JOHN J. ABEL.

(From the Pharmacological Laboratory of the Johns Hopkins University.)

This subject was taken up with the view of describing the chemical steps involved in the toxic action of acids, or more precisely stated, to determine whether the altered state of the inorganic constituents is more deleterious to muscle than the formation of a certain amount of acid-proteid compound, dissociated or non-dissociated.

When frog's muscle (gastrocnemius) is immersed in an isotonic solution of sodium chloride (100 cc.) to which five or ten cc. of a *tenth-normal* acid have been added, the problem becomes one which deals with diffusion and progressive swelling, rather than one which deals with osmotic pressure effects developed by a diaphragm which prevents the diffusion of certain newly formed substances from the interior of the muscle. This point of view is forced upon one by the following experiments. A muscle that has been immersed for two hours in an acidulated isotonic saline solution is frozen and cut. The cross section shows an outer layer more homogeneous, coagulated, and impregnated with acid and water, while the core of the muscle is normal in appearance; *i.e.*, it differs in no respect from the core of a normal muscle that

has been frozen and cut. Muscles taken from frogs that have received acid fuchsin are well adapted for the study of this diffusion of acids and the progressive formation of the watery zone, especially when the muscles are immersed in pure solutions of the acids ($\frac{1}{8}$ to $\frac{1}{10}$ normal).

Perfusion of frogs through the *bulbus aortæ* with acidulated saline solutions also gives results that lead to the above conclusion, and furthermore shows that an important rôle must be ascribed to their varying rate of diffusion when the acids are applied as in Loeb's experiments.

A muscle that has been *perfused* with an acidulated (HCl) isotonic salt solution for ten or twelve minutes is quite normal in appearance, but is little or not at all responsive to electrical stimulation and will take up little or no water when it is immersed in an isotonic acid-free salt solution. The weight-time curve in this case is practically a straight line. If, however, such a muscle is *immersed* in 50 or 100 cc. of the acid solution that was used in perfusing, it will take up water exactly as do normal muscles, although we have here no difference in the composition of the fluid within and without the muscle. Muscles that have been perfused for an hour or more and which have swelled greatly, will of course take up water less rapidly from an acidulated solution in which they are immersed. The swelling proceeds from without inwards in proportion as acid is absorbed from the outer fluid and the weight-time curve is exponential in character, the rapidity of water absorption at any given moment being proportional to the amount of muscle substance that is still capable of binding water.

A muscle that has been perfused with hydrochloric acid-saline solution for 10 minutes and which is then immersed in an isotonic acid-free saline solution for from 50 to 70 hours, produces less acid than a muscle which has been perfused with an acid-free solution of sodium chloride and which has remained for the same length of time in isotonic salt solution. The latter finally swells to a considerable extent, probably in consequence of the appearance of non-diffusible autolytic products, the development of which is inhibited in the acid-perfused muscle.

The reversed reaction, that is, the loss of water by an acid-perfused, swollen muscle when it is immersed in isotonic salt

solution, and the rate of diffusion of acid from such muscles have also been studied.

Two acids, hydrochloric and acetic, representing two extremes of electrolytic dissociation, have been compared in respect to their ability to cause swelling when distributed throughout the substance of the muscles by the perfusion method. Loeb found that these two acids among others differ greatly in their power to induce swelling of muscles immersed in saline solutions that contain these acids. Organic acids, he observed, act less powerfully as a rule than the highly dissociated mineral acids. No good explanation has been offered for this difference. Loeb found that hydrochloric acid causes muscles to take up 9 per cent of water in one hour, while acetic acid induces them to take up only 3.9 per cent in this time. A study of his data for an immersion of *eighteen hours*, shows that this ratio alters with the time of immersion, as the water intake under the influence of hydrochloric acid now attains 47.5 per cent, while under the influence of acetic acid it amounts to only 14.2 per cent. These numbers stand in the same relation to each other as the diffusion constants of the two acids. When muscles are perfused, the surfaces of contact between acid and tissue are so enormously increased that the modifying influence of the varying diffusivity of the acids should disappear. Pairs of frogs of equal weight were perfused with 100 cc. of isotonic salt solution, made up in the one case with the hydrochloric and in the other with acetic acid in equivalent amounts ($V = 100$, Loeb), one gastrocnemius being removed in each case as a control. The duration of perfusion was one hour and the amount of swelling varied from 18 to 40 per cent with both acids according to the size of the frogs used. *When the two acids are applied in this way they show no difference in their power to cause swelling in muscles of the same weight.* The muscles perfused with hydrochloric acid were quite dead, while those perfused with acetic acid still responded fairly well to electrical stimulation, thus showing that swelling and toxic action do not take a parallel course irrespective of the acid used.

Acids in general diffuse in the order of the migration velocity of their anions. When muscles are *immersed* in acidulated solution an *anion* effect may, therefore, be said to become manifest through variations in the diffusivity of the acids used. Other

f acids will be compared in the same way and the partition
sodium ion between the acid which is to be tested and that
ed from the neutral salt of the medium (NaCl or other salt)
the partition of the acids among the constituents of
will be taken into consideration. The points raised in
troduction, together with other questions pertaining to
on and dissociation will be discussed more fully in a later

W REAGENT FOR THE RECOGNITION AND ESTI-
TION OF FREE HYDROCHLORIC ACID IN GASTRIC
TENTS.

By J. H. KASTLE and H. L. AMOSS.

he Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, Washington, D. C.)

as observed by Schönbein in 1854 that the coloring matters
in flowers can be bleached by means of sulphur dioxide
e color restored by strong acids. In 1905 Kastle con-
these observations and pointed out that the affinities of
ould be determined colorimetrically by means of vegetable
g matters which had been bleached by means of sulphur
e. Evidently, therefore, such chromogens as result from
ion of sulphur dioxide on certain vegetable coloring matters
gents for the hydrogen ion. It therefore occurred to one
Kastle) that such substances might be employed as re-
in the detection and estimation of free hydrochloric acid in
tric contents. Such has been found to be the case. As a
of convenience the coloring matter of red cabbage has
mployed in the preparation of the reagent. This is pre-
by macerating the leaves of the cabbage with water, bleach-
solution with sulphur dioxide, boiling to expel the excess
hur dioxide, and filtering until clear. Sometimes small
ts of white of egg are added to properly clarify the solution.
a small amount of this solution is added to $\frac{N}{10}$ hydrochloric
to the clear filtrate from normal stomach contents a pur-
ed color is developed in the solutions. In determining the
ty of free hydrochloric acid in gastric contents 1 cc. of the
gastric contents is brought together with 2 cc. of the

reagent and 1 cc. of water. This solution is then allowed to stand 10 minutes when it is compared in a tintometer with a solution containing 1 cc. of $\frac{N}{10}$ hydrochloric acid, 2 cc. of the reagent and 1 cc. of water. In our experiments a Dubosc-Pellin colorimeter was used. In one experiment the scale of the instrument on the side of the tube containing the gastric contents was arbitrarily set at 10 divisions on the scale, the scale on the side of the instrument containing the tube with the $\frac{N}{10}$ hydrochloric acid was then found to read 4.2 divisions when the color in the two half circles of the instrument matched in depth and tint.

To find the quantity of free hydrochloric acid present in 1 cc. of the filtered gastric contents we have therefore,

$$0.00365 \times 0.42 = 0.001533$$

Hence this particular specimen of gastric contents contained 1.533 parts of free hydrochloric acid per thousand.

PHENOLPHTHALIN AS A REAGENT FOR OXIDASES AND OTHER OXIDIZING SUBSTANCES IN PLANT AND ANIMAL TISSUES.

By J. H. KASTLE.

(From the Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, Washington, D. C.)

Phenolphthalin or dioxytriphenylmethane carbonic acid is readily converted into phenolphthalein by various oxidizing agents, such as lead peroxide, potassium permanganate, potassium ferricyanide, etc. Several years ago Kastle and Shedd observed that it is also readily oxidized by the plant oxidases and recommended its use as a reagent for these substances. Since then it has been employed by a number of observers in the study of plant oxidases. Attention was called to the peculiar conduct of blood and animal tissues toward phenolphthalin. Ordinarily blood and animal tissues are incapable of oxidizing phenolphthalin. On the other hand blood and animal tissues readily oxidize phenolphthalin in alkaline solutions. In oxidizing power blood and the animal tissues thus far studied stand in the following order:

Blood	100.
Spleen	10.
Lung	8.2

Suprarenal	3.
Liver	2.5
Kidney	2.5
Testicle	2.5

The substances in the blood and animal tissues capable of oxidizing phenolphthalin in alkaline solution are weakened in their activity by boiling and completely destroyed by certain poisons, such as chlorine, bromine, and hydrocyanic acid, and also by incineration.

The oxidation of phenolphthalin by blood has been found to vary with the concentration of all the substances involved in the oxidation, viz: with that of the alkali, the phenolphthalin itself and with the quantity of blood present. On drying in the air the oxidizing power of blood towards phenolphthalin is gradually weakened but not destroyed.

It is the intention of the writer to continue these studies with the view of throwing light on the kinetics of this oxidation.

PROTEIN METABOLISM IN EXOPHTHALMIC GOITRE.

By PHILIP SHAFFER.

(From the Department of Experimental Pathology, Cornell University Medical College, New York City.)

The author has studied the protein metabolism in twelve typical cases of exophthalmic goitre, by a fairly complete analysis of the nitrogenous substances, and in some cases of the sulphur partition of the urine. The diets were in all cases free from meat products, and in most cases were fairly constant.

Two products of metabolism are of especial interest in the disease because of their very great deviation from the normal. These are creatin and creatinin.

In spite of the pathologically increased tissue catabolism in most of the cases, resulting in a fairly rapid loss of body weight, the amount of creatinin excreted was very low. From his own results and those of Folin and others in the literature, the author has concluded that a normal healthy individual excretes from 20 to 30 milligrams of creatinin per day for each kilo of body weight; and this factor the author has termed the "creatinin coefficient" (*i. e.*, number of milligrams of creatinin per kilo body weight).

The creatinin coefficients in these cases varied from 7 to 12 (one mild case being 16.8), or were less than half the normal. This fact is accepted as evidence that creatinin is not a product of total tissue catabolism, but is a product of certain *normal* cell processes, which in many diseased conditions may be extremely sluggish in their intensity, even though, as in exophthalmic goitre, the total tissue catabolism may be much increased. The low creatinin coefficients in all marked cases of exophthalmic goitre—subjects of which disease are especially prone to muscular weakness—are also accepted in support of the author's hypothesis that creatinin is an index of muscular tonus or of muscular and perhaps of general cellular efficiency.

Eight of the twelve cases excreted considerable amounts of creatin; in some cases even more creatin than creatinin. The amount of creatin appears to bear some relation to the severity of the condition. Reasons were given for believing that creatin in the urine is pathological (except when creatin is taken in food), and has a significance different from creatinin; aside from the chemical relationship there is as yet no experimental basis for the common assumption that creatin and creatinin have a close physiological connection. Their appearance in urine is the result of different causes.

ON THE BACTERIAL PRODUCTION OF SKATOL AND ITS OCCURRENCE IN THE HUMAN INTESTINAL TRACT.

By C. A. HERTER.

(From the Laboratory of Dr. C. A. Herter, New York.)

Observations upon skatol produced in the course of putrefactive decomposition are at present few and imperfect. This is due largely to the difficulties incidental to the certain recognition of this substance when present in small amounts. By means of a method described by Herter and Foster it is possible to detect the presence of very small quantities of skatol in a putrefactive mixture, to separate skatol from indol and to estimate the quantity of skatol present. This method is based on the use of β-naphthoquinone sodium monosulphonate and paradimethylamidobenzaldehyde (Ehrlich's aldehyde). By means of this method, studies

have been made with a view to discovering what organisms are chiefly concerned with the production of skatol and many observations have been made upon the presence of skatol in the human intestinal tract. A large number of facultative and strict anerobic organisms have been studied with respect to their ability to form skatol. The anerobes, *B. putrificus* (strain isolated by Bienstock) and one strain of the bacillus of malignant edema (obtained from Prof. Theobald Smith) were found to produce skatol in peptone bouillon, although it was not possible to determine the conditions under which skatol could be regularly obtained through the action of these bacteria. It was found that skatol is rarely present in the intestinal tract except in conditions of disease associated with intestinal putrefaction. Usually skatol is associated with indol in such conditions, but there are instances in which the intestinal contents contain little or no indol, and, relatively speaking, considerable skatol. This has been observed heretofore only in putrefactive processes associated with pronounced clinical manifestations.

THE CHEMICAL COMPOSITION OF THE LIVER IN ACUTE YELLOW ATROPHY.

By H. GIDEON WELLS.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, and the Pathological Laboratory of the University of Chicago.)

The report comprised a summary of certain of the results of a complete analysis of a liver obtained shortly after death, from a typical case of idiopathic acute yellow atrophy. The most important of the findings was the isolation, in sufficient quantity to be identified, of a considerable number of amino-acids, some of which had not previously been found free in human tissues. These were leucin, tyrosin, glycocoll, alanin, pyrrolidin-carbonic acid, glutaminic acid, aspartic acid, and lysin. Histidin was also present, but not isolated. Arginin, phenylalanin and tryptophan, although sought for, were not found. Xanthin and hypoxanthin were also found free in the liver extracts, but no adenin or guanin. Altogether over 8 grams of amino-acids were isolated from the watery and alcoholic extracts of 700 grams of liver tissue, corresponding to about 12 grams in the entire liver, which amount

probably represents but a small part of the free amino-acids that were actually present on account of the inefficiency of the available methods for separating amino-acids. On account of the relatively large quantity of amino-acids that seems to have occurred free in the liver in this case, the writer is inclined to agree with Neuberg and Richter in doubting if all the amino-acids present could have been derived from the autolysed liver cells. There was a slight decrease in the diamino-nitrogen (according to Hausmann's method), but not so great as observed by Wakeman in phosphorus poisoning of dogs. Sulphur was normal, and phosphorus increased. The amount of fat, both free and combined, was below normal.

THE APPEARANCE OF MILLON'S REACTION IN THE URINE IN THE ABSENCE OF PROTEINS, AS A CRITERION IN THE TUBERCULIN-REACTION.

By C. VOEGTLIN.

(From the Medical Clinic of the Johns Hopkins Hospital.)

Normal urine does not give under ordinary circumstances a positive reaction with Millon's reagent; unless the urine contains protein of some sort which possesses the tyrosin or phenylalanin molecule (oxyphenyl-reaction), the rose red tint to the precipitate does not appear. It was found, that whereas injections of tuberculin into normal individuals are not followed by the elimination of substances in the urine which give a positive reaction with Millon's reagent, after tuberculin injections into patients suffering from all sorts of tuberculous infections, the urines yield positive reactions in the first 24 hours after injection. The test was applied in the following manner: First, the urine was tested for proteins, the absence of which was always noted. Then the urine was treated with a solution of lead acetate, to precipitate the coloring matter. After filtration a practically colorless liquid was obtained. Millon's reagent was added drop by drop to the clear fluid until a pink color appeared. Heating the solution is not necessary. From one of the urines under examination that yielded a positive test, a small amount of tyrosin was isolated. While the possibility of other abnormal constituents in the urine of such cases giving the

reaction is borne in mind, it is believed that tyrosin is probably the substance which determines the positive outcome of this test. Still further evidence has to be collected to prove definitely this supposition, and to rule out other possibilities. It may be said that minimal quantities of a solution of tyrosin added to normal urine will yield a positive reaction, with the reagent under consideration. The test, therefore, seems to be extremely sensitive. The intention is to continue the work in this line.

A METHOD FOR THE DIRECT DETERMINATION OF HEATS OF REACTION.

By LAWRENCE J. HENDERSON and CHARLES T. RYDER.

(From the Laboratory of Biological Chemistry of the Harvard Medical School.)

Most thermochemical problems involve the determination of differences in heats of formation or heats of combustion, because they aim to determine heats of reaction, actual or hypothetical. Accordingly the need of a method for the direct determination of heats of reaction, even of those which proceed slowly, or a method in some way differential, had long been felt. The lack of such an aid to research is mainly responsible for the incorrect ideas still widely current concerning the regularities among the thermochemical data of organic substances; for here the significant differences are so small as often to fall within the limits of error of the determination, and seldom greatly to exceed them. Physiological reactions are not less subject to doubt on this score, as the recent investigations from Tangl's laboratory indicate.

The present investigation was aimed to test a new method for the direct determination of the heats of reaction in which the chemical change is a slow one. A thermostat filled with water and maintained at about thirty-nine degrees Centigrade, with a variation in temperature of not more than one or two hundredths of a degree, was set up. This apparatus differed in no way from instruments commonly in use in physico-chemical laboratories. The reaction mixture after being brought closely to the temperature of the thermostat was placed in a Dewar flask which rested on a tripod within the thermostat. A Beckmann thermometer was passed down through a glass tube inserted in the rubber stopper of the Dewar flask and projected into the reaction mixture. The

course of the temperature change, as indicated by the Beckmann thermometer, was recorded during several days. It was shown by preliminary experiments that evaporation from the flask did not occur in sufficient amount to influence appreciably the temperature of the flask. It was also shown that Newton's law of cooling holds under these circumstances, so that the rate of cooling or warming due to differences in temperature between the contents of the flask and the thermostat can be calculated. For the present apparatus this cooling amounts to less than one-tenth of the temperature difference in twenty-four hours.

In the experiments so far undertaken the tryptic digestion of casein has been studied, using concentrated solutions of casein dissolved in dilute sodium carbonate. After the first hour of the experiment the progress of the reaction as measured by the rise in the temperature is regular. There are, however, marked indications of a slowing of the process, very probably due to the retarding influence of the end products of the reaction. This is a new method for following the progress of a slow reaction, and will be further investigated in this laboratory. According to the data thus far obtained, the heat of this reaction is very small but positive, unless indeed the observed temperature change be due to secondary reactions. The magnitude of the heat of reaction probably lies between two-tenths and four-tenths of a great calorie per gram-molecule of water added.

With the aid of this method it is planned to study in detail various biochemical reactions in the hope of characterizing them more definitely than is now possible. The method will also be extended to the study of reactions of organic chemistry.

It seems certain that in this way an accuracy from ten to one hundred times greater than with the aid of differences in the heats of combustion can be attained in the measurement of reactions which involve little heat change. A peculiar advantage of the method is that moderate slowness of reaction is a favorable circumstance.

ON THE CONVERSION OF GLYCOGEN INTO GLUCOSE.

By A. E. TAYLOR.

ON THE INFLUENCE OF THE CONCENTRATION OF THE HYDROXYL IONS OF A SALT SOLUTION UPON THE PHYSIOLOGICAL EFFECTS OF ITS CATIONS.

By JACQUES LOEB.

FATTY TRANSFORMATION IN THE LIVER.

By H. C. JACKSON and L. K. BALDAUF.

THE EXISTENCE OF CHOLESTERYL ESTERS OF THE FATTY ACIDS IN GALL STONES AND THEIR BEARING UPON THE FORMATION OF CHOLESTERIN GALL STONES.

By J. G. ADAMI and OSCAR KLOTZ.

Second Meeting.

George Washington Medical College. Wednesday afternoon, May 8.

Presiding officer: The Vice-President, John J. Abel.

THE METABOLISM OF NITROGEN AND SULFUR IN PNEUMONIA.

By ALEXANDER LAMBERT and C. G. L. WOLF.

(From the Fourth Medical Division, Bellevue Hospital, and the Department of Chemistry, Cornell University Medical College, New York.)

Tables showing the results of the complete analysis of the urine in severe and fatal cases of pneumonia were exhibited.

On a non-nitrogenous diet of sufficient calorific value, 30 grams of nitrogen were eliminated. The relative excretion of urea was low. The creatinin excretion was high during the pyrexia, and fell rapidly after the crisis. This was also the case with uric acid. Very considerable quantities of creatin were eliminated during the febrile period. This also diminished, or disappeared after the crisis. Undetermined nitrogen, amounting in one case to 5.8

grams, was excreted in the twenty-four hours. The amount of neutral sulfur was six to eight times greater than that observed in normal individuals under similar conditions of diet.

A BRIEF NOTE ON A SOURCE OF ERROR IN THE USE OF A CERTAIN PETROLEUM ETHER AS AN EXTRACTING MEDIUM.

By JOHN MARSHALL.

(*From the Robert Hare Chemical Laboratory, Department of Medicine, University of Pennsylvania.*)

The fraction from a commercial petroleum ether, derived from the Pennsylvania oil field, which was obtained between 20°–50° C., was found, on evaporating at 200 cc. at room temperature immediately after distillation, to leave no residue, but on standing 30 days at room temperature in a stoppered flask with an air space of about one liter above the surface of the liquid and exposed to diffused sunlight, it was found that on spontaneously evaporating 200 cc. of it at room temperature and then over sulphuric acid, a cosmoline-like residue weighing 0.0072 gram remained. The remaining fluid was redistilled at 20°–50° C. and 200 cc. of the distillate on evaporation left no residue, but a portion of 200 cc. of the remainder of this distillate on standing in a flask, as above described, for 10 days left on evaporation spontaneously and over sulphuric acid a residue of 0.0007 gram and 200 cc. of the remaining liquid on standing 17 days left a residue of 0.0016 gram. On making a blank test with 200 cc. of that which had stood 17 days by using it in a Soxhlet extractor, containing a fat-free, paper thimble filled with washed and dried sand, for 14 hours and filtering the liquid through a fat-free filter and evaporating spontaneously and over sulphuric acid, a residue of 0.0138 gram remained. Two hundred cc. of a distillate which had been twice distilled at 20°–50° C. and had stood 215 days in a flask, as above described, left on evaporation at room temperature and at 100° C. for two hours a residue of 0.0494 gram, which at the latter temperature turned black. Nineteen hundred cc. of a distillate which had been twice distilled at 20°–50° C. and which, after distillation, had stood 217 hours, was redistilled at 20°–50° C. All but 135 cc. distilled over at the temperature stated and on evaporating this

spontaneously a yellowish, cosmoline-like residue remained which on being heated for 3 hours at 100° C. turned black and weighed 0.5062 gram. The composition of the residues was not determined. In every case of distillation care was exercised that the liquid in the distilling flask should not be in ebullition but that evaporation should occur in such a manner that the surface of the liquid remained placid. Weighings were made until constant weight was obtained. The cork stoppers employed had been previously extracted with ethyl ether. In some cases Glinsky's fractionating bulbs were employed. In every case there was an open space of about a liter above the surface of the liquid in the corked storage flask and the liquid was exposed to diffused sunlight. It is evident that a petroleum ether of this sort is not adapted for use in making extractions.

A CLINICAL METHOD FOR DETERMINING THE ALKALINITY OF THE BLOOD.

By HERMAN M. ADLER (by invitation).

(From the Laboratories of Clinical Pathology and Biological Chemistry of the Harvard Medical School and the Boston City Hospital.)

The methods used for the determination of the alkalinity of the blood have in the past been based on titration. These have been inaccurate because they yield information regarding only the absolute quantity of acid or alkali present and none regarding the equilibrium between the two. Since the work of Salm on the exact point of H ion concentration at which a large number of indicators turn, it has been possible to select certain indicators whose turning points correspond to the concentration of the blood. Rosolic acid seemed to be the best of these. By preparing filter paper with 0.1 per cent solution of rosolic acid, the test could be applied clinically to the serum from a large number of cases. It was found that the blood was maintained at about $2-3 \times 10^{-7} n$ H in health. In the terminal stage of acidosis a distinct variation towards the acid color was observed. In coma from meningitis no such change was observed. The interdependence of H ionization and CO_2 content of the blood has been shown by Henderson and Black; hence this method is also a measure of the latter quantity.

CALCIUM METABOLISM IN A CASE OF MYOSITIS OSSIFICANS.

By A. E. AUSTIN.

(From the Medical Chemistry Laboratory of Tufts College and the Corey Hill Hospital.)

From the literature the following principles are presented as a standard of normal metabolism of calcium. Calcium equilibrium has been maintained with an intake of 0.688–0.860 gram of calcium daily, reckoned as the oxide, but it is better to regard 1–1.5 gram as the daily need of every healthy adult and less than this as insufficient. No dependence can be placed upon the relation of feces lime to urine lime on account of its great variations and any results based upon urine lime alone are useless. Ordinarily 5–10 per cent of the ingested lime is found in the urine, while the actual amount of lime found in the urine varies from 0.15–0.5 gram, depending upon character of the food; that is, whether vegetable or animal food. The amount of lime eliminated in the urine may be increased by increased ingestion of water (v. Noorden), by the use of hydrochloric acid (Hammarsten), by lactic acid and sodium lactate (Rumpf), by bodily rest (Hoppe-Seyler), and by calcium-poor food (Rumpf). In disease impoverishment of the body in lime has been found in osteomalacia (McCrudden), in pernicious anemia (v. Moraczewski), in inanition (Ott), and in diabetes mellitus (Forster). Physiologically, retention of calcium can be attained by ingestion of large quantities without any harmful influences—pathologically, retention undoubtedly occurs in arterio-sclerosis. In order to determine whether a metabolism is pathological or not, we must learn how the individual complies with these conditions: Does he maintain a calcium equilibrium with 1–1.5 gram of this material in his daily food? Does he respond to a diminished intake with an increased procentual outgo of calcium; in other words, with an impoverishment of the body in lime. The individual under investigation, who had long been affected with myositis ossificans, and in whom a retention of lime was suspected, upon an ingestion of 1.246 gram calcium oxide maintained practically an equilibrium.

The lime eliminated in the urine was 0.472 gram and in the feces 0.787 gram, leaving a negative balance of 0.011 gram daily.

When, however, in a subsequent period of seven days, the intake was reduced to 0.572 gram daily, there was found urine lime amounting to 0.425 gram and feces lime to 0.473 gram, leaving a deficit of 0.327 gram daily. Under the latter condition the urine lime formed a much larger percentage of the lime ingested than under the former; an increase from 37.5 per cent to 47.2 per cent was found. In a similar investigation, Thayer and Hazen obtained approximate results but attribute them to the carbohydrate-free diet which was employed. Their diet, however, was very poor in lime (0.625 gram daily) and their results seem also to follow the same physiological law of loss of lime in the body when insufficient lime is given. In so far as the results of this investigation go, no evidence of calcium retention in myositis ossificans was found.

HYDROLYSIS OF SPLEEN NUCLEOPROTEIN.

By J. A. MANDEL and P. A. LEVENE.

(From the Chemical Laboratory of the New York University and Bellevue Hospital Medical College, and the Rockefeller Institute for Medical Research.)

The comparative study of the chemical composition of chromatins has been directed thus far towards the study of the nucleic acid radical. It is, however, made probable today that more distinction will be found in the investigations on nucleoproteins than on the acids. In recent years the composition of nucleoproteins was studied only once by Wohlgemuth[1] on liver nucleoprotein. The results obtained by this observer were very unusual. In the present work the nucleoprotein of the spleen, containing 14.155 per cent N and 1.605 per cent P (500 grams) was subjected to hydrolytic cleavage by hydrochloric acid.

The products of hydrolysis were separated and purified by the aid of different methods and the yield calculated for 100 grams of the nucleoprotein was as follows:

Glutamic acid	25.0 grams
Leucin / Aminovalerianic acid	6.0 "

[1] *Zeitschr. f. physiol. Chem.*, xxvii, xlii, xliv, and *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, 1904.

Glycocoll / Alanin	2.0 grams
Aspartic acid	0.5 "
Prolin	not found
Phenylalanin	present
Tyrosin	1.0 gram
Lysin picrate	7.5 "
Arginin picrolonate	2.0 "
Histidin picrolonate	0.5 "
Adenin	0.4 "
Guanin	0.6 "
Cytosin	0.7 "
Thymin	0.5 "

A COLOR TEST FOR URACIL AND CYTOSIN.

By HENRY L. WHEELER and TREAT B. JOHNSON.

(*From the Sheffield Chemical Laboratory of Yale University.*)

A characteristic purple or violet-blue color is produced when uracil or cytosin is dissolved in bromine water,and the solutions treated with an aqueous solution of barium hydroxide.

The formation of the color is explained as follows: Uracil and cytosin react with bromine to form dibromoxyhydrouracil. Barium hydroxide then converts this dibrompyrimidin into isodialuric acid, which immediately undergoes a rearrangement in the alkaline solution to give the purple barium salt of dialuric acid.

The presence of guanin, adenin, thymin, isocytosin and 6-amino-pyrimidin does not interfere with the test.

Precise directions for the application of the test have been published in this journal.

THE RÔLE OF THE OXIDIZING POWER OF ROOTS IN SOIL FERTILITY.

By OSWALD SCHREINER and HOWARD S. REED (by invitation).

(*From the Laboratory of the Bureau of Soils, United States Department of Agriculture.*)

Growing roots possess well defined powers of oxidation,due principally to the activity of enzymes. The oxidizing powers can be demonstrated by the use of reagents which produce a dye upon oxidation. Alpha-naphthylamine, benzidine, and vanillin

form insoluble dyestuffs when oxidized by the roots. Phenolphthalin and aloin produce soluble dyes and the amount of color produced is proportional to the amount of oxidation accomplished. The addition of certain substances used as fertilizers promotes the oxidative activity of the roots. The powers of oxidation are greater in fertile soils and their extracts than in unproductive soils or soil extracts. The authors also pointed out the value of oxidizing processes in aiding the decomposition of vegetable matter in the soil.

THE PRODUCTS OF GERMINATION AFFECTING SOIL FERTILITY.

By OSWALD SCHREINER and M. X. SULLIVAN (by invitation).

(*From the Laboratory of the Bureau of Soils, United States Department of Agriculture.*)

The water in which seeds have germinated, although containing nutrient salts, is by no means as good a culture medium for the seedlings as is carbon-filtered distilled water. Even the water in which seedlings have grown restrains the development of a second crop planted therein. Apparently something soluble in water and active in extremely dilute solutions accumulates in the medium in which plants have germinated.

Considering that the juice expressed from germinating seedlings would represent a concentrated extract of the toxic material present in the medium in which seeds have germinated, experiments were made on the juice expressed from wheat which had germinated seven to ten days. This juice was pale yellow, became darker on exposure to air, was acid in reaction and was free from microörganisms. If not too highly diluted it is toxic to growing plants both in water and soil, and injures likewise the resting seed. This juice acts in two ways, first, by inhibiting the life activity of the germinating seed, leading to a diminished amount of leucin and tyrosin, and to a lessening of the oxidation by the roots; secondly, by diverting the metabolism somewhat.

On analysis the expressed juice was found to contain a trace of cholin, xanthin bases and soluble organic phosphorus compounds. Whether the toxic action of the juice is due to alkaloids, such as cholin and neurin, to complex organic acids as phytic or

nucleic, to simpler organic acids, or to anti-enzymes or to a combination of all these is yet to be determined.

SOLUTION TENSION AND TOXICITY IN LIPOLYSIS.

By RAYMOND H. POND.

(*From the Chemical Laboratory of the New York Botanical Garden.*)

An effort has been made to ascertain whether lipolysis is affected by toxic agents in a manner corresponding to that found by Mathews[1] for fertilized eggs of Fundulus, by McGuigan[2] for diastatic activity and by Caldwell[3] for proteolytic digestion. Mathews announced as a general law that the toxic action of cations as such and of anions as such is an inverse function of and is determined by their solution tension. The toxic action of any salt is then an inverse function of the decomposition tension of that salt which is the sum of the solution tension of the cation and of the solution tension of the anion of that salt both values being regarded as having the same sign.

An examination of Mathews' evidence suggested the desirability of additional data and since his law had not been tested for lipolytic reaction, a study of its applicability to the saponification of ethyl butyrate by a commercial extract of pancreas has been made. The selection of this product proved fortunate because a solution can be prepared which still has lipolytic power though is too dilute to be coagulated by boiling. The enzyme activity can be determined accurately by the volume of potassium hydroxide required to neutralize the acid arising from the saponification. There is nothing to obscure the end point in titration and the relative toxicity of a series of salts can be very satisfactorily determined.

My results differ from those obtained by others, notably in the relative toxicity of silver and mercury. The latter was much more

[1] Mathews, A. P.: "The Relation between Solution Tension, Atomic Volume, and the Physiological Action of the Elements." *Amer. Jour. of Physiol.*, x, pp. 290–323, 1904.

[2] McGuigan, Hugh: "The Relation Between the Decomposition Tension of Salts and their Antifermentative Properties." *Amer. Jour. of Physiol.*, x, pp. 444–451, 1904.

[3] Caldwell, J. S.: "The Effects of Toxic Agents upon the Action of Bromelin." *Botan. Gazette*, xxxix, pp. 409–419, 1905.

toxic, while Mathews, McGuigan and Caldwell all found silver to be more toxic. The relative toxicity of a series of metallic salts is not constant with varying concentration of the enzyme as Caldwell found. The salts seem to fall into two groups with regard to the possible cause of toxicity. In one group there is evidence of chemical action between the salt and the substance of the enzyme solution and in the other group there is no evidence of such reaction. My own results considered in connection with those obtained by the men mentioned, justify the statement that the evidence upon which Mathews's law is based, is insufficient.

ON THE CAUSE OF A RED COLORATION IN THE IODOFORM TEST FOR ACETONE WHEN APPLIED TO DISTILLATES OBTAINED FROM URINE PRESERVED WITH THYMOL.

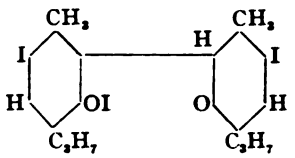
By WILLIAM H. WELKER.

(*From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.*)

In applying to urinary distillates the iodoform test for acetone, a pink to red coloration was observed in some instances. Dr. Foster in this laboratory also independently noticed this coloration. All these observations were made on specimens of urine from pathological cases. The conditions under which the coloration was produced were such as seemed to eliminate the preservative (thymol) as the cause. A study of biochemical literature on the acetone test failed to give any clue to the reason for the red coloration.

On the assumption that the compound that gave the colored substance in the test was a product secreted during disease, considerable work was done to ascertain the nature of the substance. On isolating it, the compound proved to be thymol. In spite of its apparent removal (by filtration) from the preserved urines that were subjected to distillation, thymol had nevertheless always been present *in solution* in appreciable quantity and accumulated in the distillates in proportions sufficient to permit of its identification. It was found that thymol was alone responsible for the coloration noted. On referring to the chemical literature

on thymol, it was found that Messinger and Vortmann[1] described a synthetic iodothymol compound that obviously was identical with the product formed in the test alluded to and which evidently accounted for the coloration observed. The nature of the compound is indicated by the following formula ascribed to it by Messinger and Vortmann:



ON THE GLYCOL FATS AND THE CHEMICAL AND PHYSICAL RELATIONSHIP OF CROSS FATS.

By R. F. RUTTAN (by invitation).

Third Meeting.

George Washington Medical College. Thursday morning, May 9. Joint session with the American Physiological Society.

Presiding officers: The President of the American Physiological Society, William H. Howell, and the Vice President of the American Society of Biological Chemists, John J. Abel.

ON THE OCCURRENCE OF FERMENTS IN EMBRYOS.

By WALTER JONES and C. R. AUSTRIAN.

(*From the Laboratory of Physiological Chemistry, Johns Hopkins University.*)

We have already called attention to the fact that the distribution of the nuclein ferments (guanase, adenase and xanthoöxidase) is different for different animal species. This was found notably true in the case of the livers of the four species, dog, ox, pig and rabbit, each gland being characterized and easily distinguishable from the other three by the ferments which it exhibits. This naturally suggests that the ferments in the adult

[1] Messinger and Vortmann: *Ber. d. deutsch. chem. Gesellsch.*, xxii, p. 2316, 1889.

glands of a given species will differ from those of the embryo. We have found this to be true in the case of pig's liver. The livers of small embryos (65–75 millimeters) do not contain any of the ferments. When the embryo has reached a length of 150–175 millimeters, adenase makes its appearance, but xanthoöxidase cannot be demonstrated. The adult liver as formerly stated contains both adenase and xanthoöxidase but not guanase.

PROTEIN METABOLISM IN CYSTINURIA.

By C. G. L. WOLF and PHILIP A. SHAFFER.

(*From the Chemical Laboratory and the Department of Experimental Pathology, Cornell University Medical College, New York City*).

Metabolism experiments upon a case of cystinuria. The results wholly confirm the findings of Alsberg and Folin, that the sulfur of hair- or protein-cystin fed to a cystinuric individual is normally oxidized to sulfuric acid; and are directly contradictory to the conclusion of Loewy and Neuberg, that cystinuric individuals are unable to oxidize ingested cystin. Both cystein and cystin prepared from the patient's urine were likewise oxidized when given by mouth. The cystin excreted in the urine is evidently not absorbed as such from the intestine, but must be absorbed in the form of a larger molecule; because cystin absorbed as such is oxidized. An increase of food protein leads to an increase of cystin excreted but when the food protein is hydrolyzed outside the body and the isolated cystin is given to a cystinuric patient, the sulfur of this cystin is oxidized to sulfuric acid.

Cystin injected subcutaneously was excreted in the form of neutral sulfur (probably as cystin). Cystein similarly injected led to an increase of the total sulfur of the urine, the increase being equally divided between inorganic sulfates and neutral sulfur.

The authors believe that the cystin excreted by subjects of cystinuria has a double source. A part, and perhaps a greater part on the usual diet, is derived directly from the food protein, and is therefore strictly exogenous. But a second part of the cystin appears to be independent of the food protein, and is therefore not exogenous. At least one phase of the anomaly in cystinuria appears to consist of the inability to oxidize that part of the sulfur-containing protein which has not been split so far as

the cystin stage in the intestine. That part which is absorbed as cystin the cystinuric, as well as the normal individual, does oxidize.

PROTEIN METABOLISM IN THE DOG.

By C. G. L. WOLF.

(*From the Department of Chemistry, Cornell University Medical College, New York City.*)

The nitrogen and sulfur metabolism in dogs in an early stage of starvation was examined. The animals were fed on a non-nitrogenous diet of fat and carbohydrates containing 80 calories per kilo. At the end of eight days a large quantity of protein in the form of casein was administered, and the metabolism followed during four days of subsequent starvation. In a second series, 160 calories per kilo were fed at the end of the 80 calorie period. This was done in an attempt to change the distribution of nitrogen and sulfur as a consequence of the high caloric value of the diet. The total nitrogen excretion was reduced to a level not heretofore observed in dogs of this weight. The ammonia nitrogen was relatively increased as a result of the starch and fat diet. The oxidized sulfur was lower markedly, both absolutely and relatively. The relative excretion rose at once on the administration of the protein. The absolute creatinin excretion was uninfluenced by any change in diet. The undetermined nitrogen and neutral sulfur were increased with the administration of protein, but decreased relatively to the nitrogen and sulfur outputs, respectively. There was no constant relation between the elimination of ethereal sulfur and indican.

ON THE GLOMERULAR EXCRETION UNDER CERTAIN CONDITIONS.

By A. B. MACALLUM.

(*From the Physiological Laboratory of the University of Toronto.*)

When large quantities of distilled water (2–3 liters) were swallowed in the interval of one hour and a half and the characters of the urine of each ten minute period were determined it was found that Δ progressively diminished until it was only 0.09. At

this point the sodium chloride was only 0.047 per cent. Potassium salts were not present and only traces of phosphates were found. The results seem to show in this case that the reflected epithelium of Bowman's capsule actively removes only pure water from the blood stream through the glomeruli and that the salts found in it ultimately are those washed out from the epithelial cells of the tubules, ureters and bladder. The water, therefore, of urine cannot be wholly the result of pressure filtration merely but must be also a true secretion.

ON THE COMPOSITION OF THE HOURLY EXCRETION OF URINE.

By C. C. BENSON.

(From the Physiological Laboratory of the University of Toronto.)

The urine excreted hourly for twenty-four hour periods was examined quantitatively for chlorides, phosphates and nitrogen and also as to its conductivity, its specific gravity and the depression of its freezing point. As a result of these observations it was found that while the constants of Bugarszky's and Koranyi's formulae were exemplified in some of the hourly excretions they were not uniformly or even generally obtained.

THE INHIBITION OF TETANY PARATHYREOPRIVA BY EXTRACTS OF THE PARATHYROID GLAND.

By S. P. BEEBE.

(From the Department of Experimental Pathology, Cornell University Medical College, New York.)

The acute symptoms following the extirpation of the parathyroid gland in dogs have been completely inhibited by the hypodermic administration of the nucleoproteid of beef parathyroid. The symptoms may be alleviated for three days by a single injection, but it has not been possible to prevent ultimate death by the continued injection of this proteid. If the alkaline solution of the proteid is first boiled it fails to act. The active principle is not destroyed by the peptic or tryptic digestion for forty-eight hours.

PROTEID SUSCEPTIBILITY AND IMMUNITY.

By VICTOR C. VAUGHAN.

(*From the Hygienic Laboratory, University of Michigan.*)

All the proteids with which we have worked, and this includes bacterial, animal and vegetable proteids, can be split up by dilute alkali in absolute alcohol into poisonous and non-poisonous portions. The poisonous part, which is similar but not identical in the different proteids, is freely soluble in absolute alcohol and in water, more freely in the former than in the latter. It gives the general proteid color reactions with the exception of that of Molisch, and it does not contain a carbohydrate group. It is also free from phosphorus. From its alcoholic solution, it is precipitated by ether. Its aqueous solution is distinctly acid to litmus and slowly decomposes sodium bicarbonate. The free poison when administered intra-abdominally, subcutaneously or intravenously kills guinea pigs in a few minutes. At first the animal shows evidence of peripheral irritation, then it becomes partially paralyzed, and finally it develops violent convulsions that terminate in death.

Guinea pigs can be sensitized to any of the proteids with which we have worked. In case of the vegetable and animal proteids the first dose has no recognizable effect on the animal, but the second dose, provided that there has been a proper time interval which varies somewhat with the different proteids, profoundly affects and may kill the animal. The symptoms induced by the second dose of the unbroken proteid in a sensitized animal are identical in character and sequence with those induced by the first dose of the free poison. It is evident from this that the sensitized animal splits up the proteid to which it has been sensitized with the liberation of the poison, much as the proteid may be broken up in the retort with the alcoholic solution of alkali. Undoubtedly this cleavage is carried out much more efficiently in the animal body than in the retort, and in the body it is most likely due to the action of a specific proteolytic ferment, which is called into existence by the first injection of the proteid, is stored in the animal cell as a zymogen, and is activated by the second injection.

The non-poisonous portion of the split proteid contains all the phosphorus present in the unbroken body, and when the original proteid possesses a carbohydrate group this remains in the non-poisonous split product. The non-poisonous portions of some bacterial proteids immunize animals to living cultures of homologous bacteria, and that of some vegetable and animal proteids sensitizes animals to the proteids from which they are derived. Hypersusceptibility or anaphylaxia, as it has been called, and immunity, although apparently antipodal, are in reality identical in their essentials. In both there is developed in the animal body the capability of splitting up the specific foreign proteid. If the foreign proteid be a living one—a bacterial cell—and the specially prepared ferment splits it up before it has time to multiply, the animal is immunized and its life is saved. If the foreign proteid be a dead one, and if it be present in sufficient quantity to furnish a fatal dose of the poison on being split up, the animal dies.

ON THE CHEMICAL RELATION BETWEEN COLLAGEN AND GELATIN.

By A. D. EMMETT and WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

We have been unable, by Hofmeister's method of continuous drying at 130° C., to convert either pure gelatin or commercial gelatin to collagen. The resultant desiccated products were somewhat less soluble than the original gelatin both in water and in dilute sodium carbonate solutions at 40° C., but were digested with apparently the same readiness in neutral trypsin solution at 40° C., in which even very minute fragments of collagen fibers remained unaffected for hours.

It appears that Hofmeister attached too much significance to the difference in solubility between the unmodified gelatin and the desiccation product. The observed difference may have been due to *decomposition* instead of to simple *dehydration*. Besides, he did not apply the tryptic digestion test to his products to ascertain whether they resembled collagen in resistance to tryptolysis.

When fresh tendon, ossein shavings, and pure collagen from bone and tendon were boiled in water about two hours for the production of gelatin, considerable ammonia was liberated from each. *When gelatin was subjected to the same treatment, ammonia was not eliminated.*

We believe that the so-called collagen obtained by Hofmeister from gelatin by desiccation at 130° C. was not collagen, and that his conclusion and the prevalent view that gelatin is a simple hydrate of collagen are not well founded. That gelatin arises from an intramolecular rearrangement of collagen on treating the latter with boiling water and that the resultant gelatin is not a simple hydrate of collagen, are shown by the fact that ammonia is liberated from the collagen when the latter is converted into gelatin.

EMBRYO-CHEMICAL STUDIES—THE PURIN METABOLISM OF THE EMBRYO.[1]

By LAFAYETTE B. MENDEL.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*)

In connection with a more extended series of chemical investigations in embryonic growth conducted by the writer, Dr. P. H. Mitchell has studied the occurrence and development of the enzymes associated with the metabolism of the purins. The more important conclusions already reached may be summarized as follows:

1. The liver of the embryo pig contains *adenase* but no *guanase*. In this respect it resembles the adult liver of the same species.

2. An extract of embryo viscera exclusive of the liver readily indicates the presence of guanase.

3. The view that guanase and adenase have an unlike distribution and are therefore specific enzymes receives new corroboration from such results.

4. Extracts of the organs of embryo pigs have failed to demonstrate any capacity for forming uric acid from free purins, even

[1] The expenses of this investigation were shared by the Carnegie Institution of Washington and the Sheffield Laboratory of Physiological Chemistry, Yale University. The data will later be published in detail.

after prolonged digestions of five days' duration in the presence of oxygen. The added purin bases, although deamidized, could be quantitatively recovered. *Xanthoöxidase* was therefore not recognized in such extracts.

5. On the other hand, extracts of the livers of adult pigs or of very young pigs, are capable of forming uric acid.

6. The *uricolytic enzyme* has not been identified in any extract of embryo organs under conditions in which its presence is easily demonstrated in adult tissues.

7. Both the xanthoöxidase and the uricolytic enzyme apparently begin to functionate either in the last stages of embryonic life or soon after birth.

The tardy appearance of some of these enzymes is of interest in view of the peculiar character of the physiological processes characteristic of young or growing organisms.

NOTES ON THE THYROID.

By REID HUNT.

THE DISTRIBUTION OF SULPHUR AND PHOSPHORUS IN THE HUMAN BRAIN.

By WALDEMAR KOCH.

Fourth Meeting.

Cosmos Club. Thursday evening, May 9. Joint session with the Washington Section of the American Chemical Society.

Presiding officers: The President of the Washington Section of the American Chemical Society, Peter Fireman, and the Secretary of the American Society of Biological Chemists, William J. Gies.

CHEMICAL AND BACTERIOLOGICAL STANDARDS NOW IN USE IN WATER ANALYSIS.

By J. H. KASTLE.

(*From the Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, Washington, D. C.*)

The various chemical and bacteriological standards now in use in water analysis were reviewed and discussed. Particular atten-

tion was paid to the recent work of Leighton on this subject,[1] in which this author shows that the results of the chemical analysis are ofttimes at variance with experience so far as the purity or pollution of a natural water is concerned. In the light of all of the facts, as they are known to us at the present, three things must be known before we can form a correct opinion regarding the purity or pollution of a natural water: (1) The source of the water and the sanitary character of the drainage area; (2) the number and kind of microörganisms present in the water, and (3) the nature and amount of the chemical impurities.

By way of illustration the several water supplies of the District of Columbia were considered and also the purification of the water of the Potomac river effected by sedimentation and sand filtration, as shown by the chemical and bacteriological findings. Special attention was called to the parallelism found to exist between the turbidity of the Potomac water at various stages in its purification by sedimentation and sand filtration, and the number of bacteria per cubic centimeter and also to the parallelism between the several amounts of nitrites and the number of specimens of water showing the *B. Coli*.

AMMONIA IN MILK AND ITS DEVELOPMENT DURING PROTEOLYSIS UNDER THE INFLUENCE OF STRONG ANTISEPTICS.

By H. C. SHERMAN, W. N. BERG, L. J. COHEN and W. G. WHITMAN.

(*From the Department of Chemistry, Columbia University.*)

Analyses of 28 samples of milk obtained from 7 different sources showed an average of 0.0004 per cent of ammonia. As a rule the better the milk and the fresher when examined the lower were the percentages of ammonia found.

Addition of 3 per cent chloroform or 0.1 per cent formaldehyde retarded but did not stop the proteolysis which resulted in the production of ammonia. The greater the freedom of the milk from contamination the less apparent is this influence of the antiseptic and in one sample of exceptional purity spontaneous sour-

[1] See *Biological Studies by the Pupils of William Thompson Sedgwick*, Boston, pp. 36–53, 1906.

ing appeared to inhibit the production of ammonia to a greater extent than did the chloroform or formaldehyde.

STUDIES ON APPLE JUICE.

By H. C. GORE.

(*From the Bureau of Chemistry, United States Department of Agriculture.*)

Successive analyses were given of the juices of the cull apples employed for cider making. These juices were found to become gradually richer in sugar and acid as the season progressed. Analyses of the juices of standard varieties of apples grown in Nebraska were given, and compared with analyses taken from the literature, of apples grown in Eastern States. The composition of the Nebraska apple juice was found to be practically the same as that of Eastern grown apples.

Summer apples were discussed in regard to their value as vinegar stock. To secure the highest yield of acetic acid in the vinegar it was found that the alcoholic fermentation should be carried on in closed vessels in order to hinder the development of acetic acid bacteria which interfere with the alcoholic fermentation. Juices of decaying apples were examined with regard to their use as vinegar stock. It was found that changes go on when apples decay similar to those which occur in vinegar making, except that the inversion of the sucrose, the alcoholic fermentation and the acetic acid fermentation go on more or less simultaneously. Acetic acid was found in the juices of decayed apples and in view of the well known tendency of acetic acid to retard alcoholic fermentation the use of decayed apples is to be avoided. The question of the wholesomeness of vinegar prepared from decayed apples was not touched upon.

SUGAR METABOLISM.

By HUGH McGUIGAN.

(*From the Physiological Laboratory of the University of Chicago.*)

Work on the oxidation of sugars *in vitro* and their metabolism in the body corroborates the clinical assertion that levulose is more easily oxidized than glucose, and that it may be used in the body when glucose cannot. The order of their ease of oxidation *in*

vitro and in the economy is: levulose, galactose, glucose, maltose, saccharose, levulose being the easiest oxidized.

In cases of perverted metabolism with glycosuria my results favor the theory that the sugar in the blood normally exists in combination with a colloid. An abnormal breaking down of this compound causes glycosuria. The intravenous injection of calcium salts inhibits the glycosuria by forming a more stable compound, probably $Ca < \begin{smallmatrix}\text{proteid}\\ \text{sugar}\end{smallmatrix}$ or $Ca < \begin{smallmatrix}\text{proteid}\\ \text{glycogen}\end{smallmatrix}$. The various tissues of the body also differ in their power of oxidation. By using the volume of O evolved from H_2O_2 by the same weight of the different tissues, as an index of their oxidizing power the order found was: kidney, liver, lungs, spleen, pancreas, muscle, brain.

Preliminary work on the oxidation of sugars by the tissues gives substantially the same order, but as yet it is not conclusive.

THE PRESENCE OF SECONDARY DECOMPOSITION PRODUCTS OF PROTEIDS IN SOILS.

By OSWALD SCHREINER and EDMUND C. SHOREY.

(*From the Laboratory of the Bureau of Soils, United States Department of Agriculture.*)

Soil as distinguished from the disintegrated rock which forms the basis of soils contains organic matter. A portion of this organic matter is made up of nitrogenous compounds, which are evidently decomposition products of proteids.

We have absolutely no definite knowledge of the composition or constitution of these bodies and many difficulties are encountered in attempting to isolate them.

A method finally adopted is essentially solution in 2 per cent sodium hydroxide at room temperature, removal of mineral bases and so-called humus bodies by neutralizing the solution so obtained, resulting in a neutral solution containing 50–60 per cent of the organic nitrogen in the soil. This solution gives precipitates with silver nitrate and ammoniacal lead acetate and by decomposing the precipitate so obtained with hydrogen sulphide, a crystalline body has been obtained which has been identified as picoline carboxylic acid.

This compound has been obtained in small quantity from several soils of widely different character.

The presence of this pyridin compound may be connected with the following facts. The melanoidins or humus bodies yield pyridin on reduction. Tryptophan, a constant decomposition product of proteids, can be easily converted into a pyridin derivative. Pyrouvic acid on treatment with alcoholic ammonia yields uvitonic acid which on heating splits up into carbon dioxide and picoline carboxylic acid.

There have been obtained indications of the presence of pyrouvic acid in some soil solutions but the identification is not as yet conclusive.

The work here outlined is the initial stage of one phase of the investigation of the relation of organic soil composition to soil fertility now being carried on by the Bureau of Soils and a more detailed account of the work will be published at a later date.

ON LYSYLGLYCIN.

By P. A. LEVENE and W. A. BEATTY.

(From the Rockefeller Institute for Medical Research.)

In the process employed by the writers a year ago for preparing the peptid prolinglycyl, a substance was produced by tryptic digestion of egg albumen, which on further cleavage yielded only lysin and glycocoll. The substance could not be crystallized. It is a noteworthy fact that peptids of the hexon bases obtained by Fischer and Suzuki synthetically also failed to crystallize.

SOME AZOLITMIN COMPOUNDS OF MUCOIDS, NUCLEOPROTEINS AND OTHER PROTEINS, WITH EXHIBITION OF PRODUCTS.

By JACOB ROSENBLOOM and WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

It is well known that mucoid, completely freed from the acid used to obtain it, is acid to litmus. All previous references to this fact appear to depend upon reaction tests made simply with

litmus paper. We have recently made the following additional observations.

When a sample of pure moist mucoid is mixed with a small quantity of a commercial blue litmus or azolitmin product, the entire mass assumes a *raspberry red* color. We have been unable to remove this color by any washing process, although an excess of litmus may be readily eliminated by treatment with water.

Very faintly alkaline azolitmin preparations yield mucoid products that are also raspberry red when moist and which are somewhat soluble in water. If, however, one removes the excess of base by thorough dialysis in water, azolitmin forms a raspberry red product with moist mucoid that appears to be entirely *insoluble* in water.

This moist raspberry red product turns blue when treated with a trivial quantity of base, such as dry CaO, and the material then dissolves in water yielding a fine blue solution. A blue precipitate is obtained upon treating this solution with an excess of alcohol. This precipitate dissolves readily in water. Alkalies intensify the blue color of the solution of this precipitate, but acids, and even CO_2 gas, precipitate a flocculent *pink* product, which is insoluble in water and from which none of the color can be washed. This pink precipitate in turn dissolves readily to a blue solution in aqueous basic liquids.

Similar experiments are under way with other coloring matters and additional proteins, and pure products will shortly be made for intimate chemical study. We believe that mucoid, and proteins such as nucleoproteins that behave in the same way, form definite compounds with azolitmin. That the phenomena observed are not adsorption effects is already indicated, although not conclusively proved.

NEGATIVE EVIDENCE OF THE ADAPTATION OF DOG'S SALIVARY SECRETION TO MEET THE DIGESTIVE REQUIREMENT OF THE DIET.

By WALTER E. GARREY.

(*From the Physiological Laboratory of the Cooper Medical College.*)

1. In testing the salivas of forty-seven dogs fed upon a mixed diet it was found that only four showed any diastatic action

upon starch paste of from 1 to 5 per cent strength. Only about 8 per cent of dogs, therefore, secrete an active diastatic enzyme in their saliva, and these only in very small amounts.

The samples of saliva were obtained in several ways; permanent fistulas being established in some cases, in others drippings were obtained under the influence of various stimuli, *e. g.*, the "psychic" secretion, that obtained by pilocarpine injections, by induction of nausea, by sensory stimulation (both mechanical and chemical), and by faradic excitation of the glandular nerve supply (parotid and submaxillary), the animal being under morphine or chloretone anesthesia. Eleven hundred samples of saliva thus obtained gave only the negative result mentioned above.

2. A diet of bread alone, of bread and milk, or of bread and bouillon failed to develop any diastatic power when it had previously been absent, or to increase the action in the few salivas which had previously shown some slight diastatic action. The carbohydrate diet was maintained in these cases from 18 to 40 days.

3. The failure of dog's saliva to digest starch paste cannot be ascribed to the fact that proferments *only* are present, for the activity is not forthcoming upon changing the reaction of the media or by adding extracts of the esophageal or gastric mucosa, nor does starch paste mixed with saliva show any reducing action upon Fehling's solution after remaining in dogs' stomachs from 20 to 45 minutes—no "activation" takes place.

4. Extracts of active and resting dog's salivary glands showed no quantitative difference in diastatic power, and this was no greater than one would expect from tissues in general.

5. Hewlett's observation that fat splitting enzymes are absent from dog's saliva was confirmed.

6. Proteolytic enzymes capable of attacking raw fibrin are also absent.

7. Diets containing much fat, or meat exclusively, do not induce the secretion of lipase or proteolytic enzymes, respectively.

8. Histologic examination shows the typical granular structure of the salivary glands of the dog. We conclude that these granules are not "zymogen" granules.

ON THE QUANTITATIVE DETERMINATION OF MUCOID IN URINE, BLOOD AND TISSUE EXTRACTS.

By CLARENCE E. MAY and WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Connective tissue mucoids cannot be completely precipitated from their neutral or alkaline solutions by any degree of acidification, however cautiously acidity may be brought about and increased. As much as 10 to 15 per cent of a given amount of mucoid, dissolved in lime water under ordinary conditions, may remain in solution after apparent attainment of complete precipitation by acid, in the usual flocculent condition in a water clear liquid. Treatment of the clear filtrate with alcohol in excess causes precipitation of practically all the remaining mucoid material.

The acid precipitation method is very unsatisfactory for quantitative determination of mucoid in tissue extracts, and biological liquids in general, not only because the mucoid cannot be completely precipitated by mere acidification, but also because other proteins that may be present are apt to be carried down with the mucoid in flocculent combinations that cannot be severed by any washing process.

Observations in other connections indicate that practically all the acid precipitable proteins are like mucoid in these several respects.

The results of numerous quantitative experiments have enforced the above conclusions and make it apparent that all published data for the proportions of mucoids in tissues or biological liquids are inaccurate. We hope to devise a new method for the more accurate quantitative determination of mucoids and other acid precipitable proteins.

ON THE NATURE AND OBJECTS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

By WILLIAM J. GIES (by invitation).

ON THE FRACTIONATION OF AGGLUTININS AND ANTITOXIN.

By ROBERT BANKS GIBSON and KATHARINE R. COLLINS.

(*From the Research Laboratory of the Department of Health, of the City of New York, William H. Park, M.D., Director.*)

(Received for publication, May 31, 1907.)

E. P. Pick[1] in 1901 associated a number of antisubstances individually with the one or the other of the two serum globulin fractions of the Hofmeister classification. In the pseudoglobulin (3.4 to 4.6 saturated ammonium sulphate solution)[2] group of antibodies he placed the diphtheria and tetanus antitoxins and the typhoid agglutinin of horse serum; the lower or euglobulin fraction (2.9 to 3.4 saturated) comprises diphtheria and tetanus antitoxin and cholera lysin in the goat, rabbit and guinea pig, and finally cholera agglutinin in the horse and goat. It becomes possible, according to Pick, to separate the individual specifically reacting antisubstances by fractioning appropriate mixtures of sera. Such a possibility suggested the application of this method to the further study of certain antibodies, especially of the relation of specific and group agglutinins developed by immunization against a single strain of organism. Preliminary experiments in the course of our investigation indicated the unreliability of Pick's differentiation, and attention was accordingly directed to the actual possibility and practicability of distinguishing between antibodies by fractionation of the globulin. The availability of polyagglutinative sera for the work gave a chance for making numerous and extended observations of the distribution of these antibodies in the fractions.[3]

[1] *Beitr. z. chem. Physiol. u. Path.*, i, p. 351, 1901.

[2] The degrees of saturation, as here expressed, indicate a concentration equivalent to a content in 10 cc. of the precipitated solution of 3.4 and 4.6 cc. of saturated ammonium solution respectively.

[3] A preliminary account of our results was published several months ago in the *Proceedings of the Society for Experimental Biology and Medicine*, iv, p. 15, 1906–1907.

The literature on the fractional precipitation of the antibodies is not extensive. Ide and Lemaire[1] (in 1899) found the precipitation limits of diphtheria antitoxin in horse serum to be from 2.8–4.4 saturation. Fuld and Spiro[2] (1900) associated the antirennin of horse serum with the pseudoglobulin, while a milk-coagulating action was possessed by the eu-fraction. The failures of Brodie and of Atkinson (of this laboratory) to separate diphtheria antitoxin from the accompanying serum globulins are referred to in the following paper. Porges and Spiro[3] without giving any of their experimental protocols divide, according to the distribution of the antibodies, the serum globulin into three distinct fractions; the ammonium sulphate precipitation boundaries of these overlap unless the serum is greatly diluted. Landsteiner[4] found that the antitryptic action of blood serum is possessed by the albumin precipitated by complete saturation with ammonium sulphate after removal of the globulin. Cathcart[5] also observed the antitrypsin to be associated with the albumin but not with the globulin fraction. Glaessner[6] states that the euglobulin fraction inhibits the action of trypsin, but the typical protocol which he publishes and his statement of the Hofmeister classification show a misconception and confusion of the identity of his fractions.[7] Glaessner apparently found that the globulin remaining in solution on dialysis was antitryptic.

Very recently Simon, Lamar and Bispham[8] found that the opsonic substance in blood serum was precipitated with the serum

[1] Ide and Lemaire: *Arch. internat. d. pharmacodyn.*, vi, p. 477, 1899.

[2] Fuld and Spiro: *Zeitschr. f. physiol. Chem.*, xxxi, p. 133, 1900.

[3] Porges and Spiro: *Beitr. z. chem. Physiol. u. Path.*, iii, p. 277, 1903

[4] Landsteiner: *Centralbl. f. Bakt.*, xxvii, Abt. I, p. 357, 1900.

[5] Cathcart: *Journ. of Physiol.*, xxxi, p. 497, 1904.

[6] Glaessner: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 79, 1904.

[7] The error on his part has not heretofore been noted. Glaessner states (p. 82): "Das Globulin des Blutserums lässt sich nach den in Hofmeisters Laboratorium in mindestens 3 Fractionen zerlegen: in das bei 25 Proz. Sättigung mit Ammonsulfat ausfällbare Fibrino-globulin, in das Euglobulin, das bei einer Sättigung von 33 Proz. ausfällt und bei der Dialyse in Lösung bleibt, und endlich in das bei 38 Proz. Sättigung ausfällbare bei der Dialyse unlösliche Pseudoglobulin." The confused nomenclature of the degrees of salt saturation is discussed in the following paper (p. 254).

[8] Simon, Lamar and Bispham: *Journ. of Exp. Med.*, viii, p. 651, 1906.

proteids which separated out on dialysis. Opie and Barker[1] observed that proteolysis in an alkaline medium by the enzyme of the leucocytes is inhibited by the serum albumin.

In a paper just published on the relation of diphtheria antitoxin to the serum globulin, Ledingham[2] finds that the pseudoglobulin of horse serum contains the greater part of the antitoxin. The repeatedly precipitated euglobulin, however, in one horse still contained fully 10 per cent of the antitoxin; in a second horse, the euglobulin similarly treated contained practically none of the antitoxin. Single precipitations (without further purification) showed that large amounts of antitoxin may be carried down with the lower fraction; with the one horse, judging in part from the tests on the pseudoglobulin fraction, over half the units must have precipitated with the euglobulin.[3] Ledingham also confirms our own observation here reported that the diphtheria antitoxin of the goat is not invariably linked to the euglobulin fraction as maintained by Pick.

Recently observations have been made by Ruediger[4] on the relation to the blood proteids of streptolysin, the hemolytic substance produced by the development of streptococci in heated serum. The lysin was precipitated with the globulin by saturation with magnesium sulphate; it was found with both the euglobulin and pseudoglobulin of the fractioned undiluted serum and also in both the insoluble proteid and the filtrate of the dialyzed half-

[1] Opie and Barker: *Journ. of Exp. Med.*, ix, p. 207, 1907.

[2] Ledingham: *Journ. of Hygiene*, vii, p. 65, 1907.

[3] Brieger (*Festschrift für R. Koch*, Jena, 1903) and also one of us (Gibson) have already reported similar experiences. In some as yet unpublished experiments carried on for another purpose by one of us (Gibson) and E. J. Banzhaf of this laboratory, it has been found that if undiluted horse serum be precipitated with half its volume of saturated ammonium sulphate solution and allowed to stand for 18 hours, the euglobulin precipitate may contain over two-thirds of the total serum globulin. Precipitation under the same conditions except that the precipitated mixture is 5 or 10 times the volume of the original serum gives a euglobulin figure only of from a fifth to a third the total globulin. The euglobulin at a dilution of 1:10 is noticeably smaller than at 1:5. This fact explains the diminished antitoxic content of reprecipitated or washed euglobulin fraction and makes difficult any hard and fast division into eu- and pseudoglobulins.

[4] Ruediger: *Journ. of Infect. Diseases*, iv, p. 377, 1907.

saturation ammonium sulphate precipitate. Moll,[1] however, has shown that such heating suffices to alter the chemical composition and to change the precipitation characters of the blood proteids.

Owing to the difficulty of making a hard and fast division of the serum globulins into the eu- and pseudo-fractions, our experiments were not planned to be interpreted especially from the quantitative occurrence of the agglutinins in the one or the other fraction of specific agglutinating sera. We have aimed, however, to determine if the relative proportion of the agglutinins of polyagglutinative sera in the fractions remained constant as regards the proportional distribution of all the agglutinins of the serum in the eu- and pseudo-globulins. In a word, by a difference of precipitation limits to ammonium sulphate, it should be expected that the bulk of one or more of the agglutinins would appear in the one fraction, as contrasted with the larger proportion of each of the remaining agglutinins occurring in the other fraction. Attention is particularly directed in studying the results from our standpoint to the pseudoglobulin fraction (filtrate from the euglobulin fraction). Any loss from the euglobulin fraction through solubility in the wash solution has been considered only in the case of the antitoxins. Such loss may be interpreted as due to the resolution of the mechanically precipitated proteids of the more soluble fraction; it may be considered just as well in part as a not absolute insolubility of the eu-fraction in 3.4 saturation ammonium sulphate. The content of agglutinins in the washed euglobulin fraction is of interest, however, as it is more highly "purified" than the pseudoglobulin, so that any relative differences in the distribution of the agglutinins should be from this standpoint the more pronounced for the low fraction.[2] The limitations in determining the agglutinative potency of the serum and of the fractions, however, make difficult at times the interpretation of the readings obtained, and do not permit conclusions being drawn from a single experiment.

[1] Moll: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 563, 1904.

[2] The euglobulin of 2 cc. of serum precipitated at a final dilution of 1 : 5 was washed usually by being three times thoroughly suspended in 10 cc. of 3.4 saturation ammonium sulphate solution and recentrifuged.

It was found repeatedly in our experiments with rabbit and goat sera that the agglutinins for the dysentery group of organisms (Flexner Manila and Shiga), typhoid, colon and cholera, were not confined to either the pseudoglobulin of the washed (with 3.4 saturated ammonium sulphate solution) euglobulin fractions; they were either split by the fractioning, the larger portion occurring in the pseudoglobulin, or almost the entire amount of the agglutinating substances recovered were in this higher fraction in the original quantitative proportion to one another. With antidysentery horse serum, the dysentery (Shiga and Flexner) and *B. coli* agglutinins were fairly quantitatively split between the pseudo- and euglobulin fractions, the latter containing the lesser amount. With an anticholera and antityphoid horse serum, the pseudoglobulin (two experiments) and also the filtrates from two additional 3.6 and 3.8 saturation precipitations contained the bulk of both the agglutinins. In subsequent experiments with sera from other bleedings as well as with the sera used above, the typhoid agglutinin was divided between the two fractions with a somewhat larger proportion occurring in the pseudoglobulin.

The results of exhaustion experiments on the two globulin fractions were the same as those that would be obtained in the use of the native serum, and failed to give any reason for believing that we were dealing with a separation of group and specific agglutinins through fractioning.[1]

[1] Immunization with certain bacteria results in the development of "group" or "common" and of "specific" agglutinins in the serum of the animal immunized. Group agglutinins are agglutinating substances which are effective both on the homologous organism and on some allied strains of bacteria; specific agglutinin is effective on the organism used for immunization. An immune serum developed by immunization against *B. dysenteriæ* (Shiga) might contain, for the sake of illustration, simply (1) a group agglutinin effective for Shiga, Flexner Manila, Pfeiffer and *B. coli*, and (2) an agglutinin specific for the Shiga strain. When immunization has been developed simultaneously against two or three of the above organisms instead of the Shiga strain alone, the number and agglutinating scope of the agglutinins resulting becomes more complex, various group agglutinins and the specific agglutinins for each organism being present. The existence of the two types of agglutinins is demonstrated by the agglutinating characters of the diluted serum after the agglutinins for any desired strain of organism have been exhausted by adding sus-

Precipitation of antidiphtheria goat serum (three experiments) showed that less than half the antitoxin remained in the pseudoglobulin; practically none was found in the euglobulin while the 3.4 saturated ammonium sulphate solution washings contained the larger part. Our results with the antitoxic horse serum at a dilution of 1:5 are essentially identical with Pick's.

The results of the work accomplished have demonstrated the untrustworthiness of any such differentiation of the antibodies as those contained in the euglobulin and those of the pseudoglobulin. No evidence has been adduced from our experiments to show that the agglutinins developed in the rabbit, goat and horse can be classed as belonging to either globulin, or that these antibodies can be separated from one another by ammonium sulphate fractioning of polyagglutinative sera.[1]

A description of the experimental procedure is given with the protocols which follow:

FRACTIONATION OF POLYAGGLUTINATIVE RABBIT SERUM.

Combined immunization against Flexner Manila dysentery, Shiga dysentery, Pfeiffer,[2] colon and cholera. Rabbit bled II/27/06. Serum fractioned II/29/06.

Five cc. of the rabbit serum diluted with 11.5 cc. of distilled water, were precipitated by 8.5 cc. of saturated ammonium sulphate solution. After standing 2 hours, 12.5 cc. of the uniform mixture were removed and centrifuged in stoppered tubes. The supernatant fluid contains the "pseudoglobulin" fraction. The

pensions of washed organisms; after contact for some hours the agglutinated and excess of free organisms are removed by filtration. The agglutinins for several related strains can thus be successively withdrawn. In the example given above, exhaustion with either *B. coli*, Flexner Manila or Pfeiffer would remove only the group agglutinin (1) so that the serum would still agglutinate the Shiga, though at a diminished dilution; exhaustion with the Shiga would take out both the group and the specific agglutinins, and this serum would no longer have agglutinating properties for any of the above organisms. Cf. Castellani: *Zeitschr. f. Hyg. u. Inf.*, xl, p. 1, 1902; also Park and Collins: *Journ. Med. Research*, xii, p. 491, 1904.

[1] In the paper by Banzhaf and Gibson following this article, it will be shown that the globulins of the serum do differ markedly in their content of antitoxin per gram proteid.

[2] The original Pfeiffer strain of *B. typhosus*.

precipitate ("euglobulin") was washed three times by being thoroughly suspended in 3.4 saturated solution and centrifuged; it was once more suspended and the volume made up to 12.5 cc. The agglutinating properties were then ascertained of (1) the original serum; (2) the total globulin, a uniform sample of the precipitated serum; (3) the pseudoglobulin fraction, and (4) the washed 3.4 saturation precipitate or euglobulin fraction. Agglutinations were determined microscopically and control slides were examined. Dilutions are in terms corresponding to the original serum. The characters of the agglutinations at the various dilutions are indicated as follows:

+ + + +	agglutination with no free organisms.
+ + +	agglutination with relatively very few free organisms.
+ +	agglutination but with numerous free organisms.
+	incomplete agglutination, small loose groups and many free bacteria.
±	tendency to agglutinate.
–	no agglutination.
o	observation lost.

The procedure was essentially unchanged in the other experiments. The serum used in this and the following experiments was roughly tested for orientation before the final agglutinations were made. A + + + agglutination indicates usually the highest dilution for an observed positive reaction. It should be remembered that the character of the agglutination at any dilution is often difficult to decisively determine; *the actual observations are by no means so exact as would be inferred from the published experiments of Pick and of others.*

The agglutinating properties of this rabbit serum (Table I) were too low to be entirely satisfactory for fractioning, the weakest agglutinating action (1:50) being manifested on the Shiga strain. The agglutinin for this organism drops out in the euglobulin fraction. This lost agglutinin is not found in the pseudo fraction. It is conceivable from this experiment that the agglutinin of the Shiga dysentery is more soluble than that of the other five strains; however, the Shiga shows no such differences in the two following precipitation experiments on later bleedings of the same rabbit. It is more likely that the content of serum in agglutinin was originally so low that in the fractioned and washed

euglobulin the dilution of 1:20 failed to show the proportion of agglutinin present. With the Flexner Manila dysentery, the Pfeiffer typhoid strain, the colon and the cholera—all contained in greater concentration than the Shiga agglutinin—the major portions of the agglutinins occur in the pseudoglobulin, a smaller amount being held by the low fraction.

TABLE I. FRACTIONING OF POLYAGGLUTINATIVE RABBIT SERUM.

Bleeding of II/27/06; fractioning II/29/06.

Organism.	Fraction.	20	50	100	200	500	1000
Flexner Manila..	Original serum	++++	++++	++++	**++++**	++	±
	Total gbl.	++++	++++	++++	**++++**	++	–
	Pseudogbl.	++++	++++	**++++**	+	±	–
	Eugbl.*	++++	++++	**++++**	±	–	–
Shiga ...	Serum	++++	**++++**	++	±	–	–
	Total gbl.	++++	**++++**	+	–	–	–
	Pseudogbl.	**++++**	±	–	–	–	–
	Eugbl.*	–	–	–	–	–	–
Pfeiffer ..	Serum	++++	++++	**++++**	++	±	–
	Total gbl.	++++	++++	**++++**	++	–	–
	Pseudogbl.	++++	**++++**	+	±	–	–
	Eugbl.*	++++	**++++**	+	–	–	–
Colon....	Serum	++++	++++	++++	**++++**	±	–
	Total gbl.	++++	++++	**++++**	++	±	–
	Pseudogbl.	++++	++++	**++++**	+	±	–
	Eugbl.*	++++	**++++**	+	–	–	–
Cholera...	Serum	++++	++++	++++	**++++**	±	–
	Total gbl.	++++	++++	++++	**++++**	±	–
	Pseudogbl.	++++	++++	**++++**	±	–	–
	Eugbl.*	++++	**++++**	±	–	–	–

*Tested III/1/06 with a fresh culture.

Exhaustion of the two globulin fractions (Table II) with suspensions of the Flexner Manila strain has not withdrawn the agglutinins for the typhoid from either fraction. Exhaustion with the Pfeiffer likewise is not complete for the dysentery in either pseudo- or euglobulin.

This later bleeding of the same rabbit shows by far the greater part of all the agglutinins in the pseudoglobulin (Table III), and small though relatively proportional amounts of each in the low fraction. The absolute dropping out of the Shiga does not occur as in the preceding fractioning. The results are also more uniform.

TABLE II. EXHAUSTION EXPERIMENT. POLYAGGLUTINATIVE RABBIT.

Exhaustion of the Original Serum and the Fractions (cf. Table I).

Exhaustion with Flexner Manila.

Organism.	Fraction.	20	50
Flexner Manila	Serum	+	−
	Pseudogbl.	−	−
	Eugbl.	−	−
Pfeiffer	Serum	+ + + +	+ + + +
	Pseudogbl.	+ + + +	+ + + +
	Eugbl.	+ + + +	+ + + +

Exhaustion with Pfeiffer.

Flexner Manila	Pseudogbl.	+ + + +	−
	Eubgl.	+ + + +	−
feiffer	Pseudogbl.	−	−
	Eugbl.	−	−

TABLE III. FRACTIONING OF POLYAGGLUTINATIVE RABBIT SERUM.

Serum from bleeding IV/16/06; serum fractioned IV/18/06.

Organism.	Fraction.	50	100	200	500	1000	2000
Flexner Manila	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**	+
	Pseudogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	+
	Eugbl.	+ +	+	−	−	−	−
Shiga	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**	+ +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	±
	Eugbl.	+ +	+	−	−	−	−
Cholera	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**	+
	Eugbl.	+	−	−	−	−	−
Pfeiffer	Total gbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	+
	Psuedogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+	±
	Eugbl.	±	−	−	−	−	−

The third precipitation (Table IV) shows apparently a recovery of all the agglutinins in the high globulin fraction. The pseudoglobulin dilutions for the Flexner Manila, in fact, were read slightly higher than were those of the total globulin.

Pick's single observation by the test-tube method on typhoid rabbit serum, agglutinating at 1: 20,000, gave the limit of agglutination of the pseudoglobulin at 1: 3000, of the euglobulin 1:20,000; after reprecipitating the fractions three and four times, respectively, the limits of dilution for agglutination were at 1:20 and 1:8000.

TABLE IV. FRACTIONING OF POLYAGGLUTINATIVE RABBIT SERUM.

Serum from bleeding on V/23/06.

Organism.	Fraction.	50	100	200	500	1000
Flexner Manila .	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
	Eugbl.	–	–	–	–	–
Pfeiffer...	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
	Eugbl.	–	–	–	–	–
Cholera...	Total gbl.	+ + + +	+ + + +	+ + + +	+ + +	+ +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	+ + +	+ +
	Eugbl.	±	±	±	–	–

POLYAGGLUTINATIVE GOAT SERUM.

Immunization against Flexner Manila, Shiga, Pfeiffer, cholera and *B. coli*.

The proportion of the agglutinins for each organism is much greater in the pseudoglobulin than in the euglobulin fractions (Table V). This conclusion is confirmed for the Shiga, Pfeiffer and cholera by the redetermination of the agglutinations in the exhaustion experiment (Table VI). The Flexner Manila dysentery strain has not exhausted the agglutinins for the other organisms completely from the eu- or from the pseudoglobulin fractions; nor have the agglutinins apparently been withdrawn to a relatively greater degree from the one than from the other fraction.

Fractioning of the polyagglutinative serum (of lessened agglutinating power) from the second bleeding of the goat immunized against the mixed cultures showed that the agglutinins were almost quantitatively contained in the pseudoglobulin fraction (Table VII).

TABLE V. FRACTIONING OF POLYAGGLUTINATIVE GOAT SERUM.

Serum from bleeding II/27/06; fractioned III/9/06.

Organism.	Fraction.	50	100	200	500	1000
Flexner Manila	Serum	+ + + +	+ + + +	+ + + +	**+ + +**	+ +
	Total gb.	+ + + +	+ + + +	+ + + +	**+ + +**	+
	Pseudogbl.	+ + + +	+ + + +	**+ + +**	+ +	−
	Eugbl.	+ + + +	**+ + +**	−		
Shiga	Serum	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +
	Total gbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+
	Pseudogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	−
	Eugbl.	+ + + +	**+ + +**	−		
Pfeiffer...	Serum	+ + + +	+ + + +	+ +	+ +	+
	Total gbl.	+ + + +	+ + + +	+ + +	**+ + + +**	±
	Pseudogbl.	+ + + +	**+ + + +**	+ +	+	±
	Eugbl.	**+ + + +**	+	−		
Cholera...	Serum	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**
	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**
	Pseudogbl.	+ + + +	+ + + +	**+ + + +**	+ +	±
	Eugbl.	+ +	+	+		
Colon	Serum	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**
	Total gbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+
	Pseudobgl.	+ + + +	+ + + +	+ + + +	**+ + + +**	±
	Eugbl.	**+ + + +**	+ +	−		

TABLE VI. EXHAUSTION EXPERIMENT. POLYAGGLUTINATIVE GOAT SERUM.

Exhaustion with Flexner Manila of Fractions (Table V) on III/7/06.

Fraction.	Organism.	20	50	100	200	500	1000
Total gbl.	Flexner Manila	**+ + + +**	−	−	−	−	−
	Shiga	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**
	Pleiffer	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**
	Cholera	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**
Pseudogbl.	Flexner Manila	−	−	−	−	−	−
	Shiga	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	
	Pfeiffer	+ + + +	+ + + +	+ + + +	**+ + + +**	+	
	Cholera	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**	
Eugbl. ...	Flexner Manila	−	−	−	−	−	
	Shiga	+ + + +	**+ + +**	+	−	−	
	Pfeiffer	+ + + +	**+ + +**	−	−	−	
	Cholera	+	+	−	−	−	

Pick's corresponding experiment may be summed up as follows: An antityphoid goat serum agglutinating at 1:2600 was precipitated in a series of progressively increasing concentrations of ammonium sulphate. The initial precipitation was at 2.6 saturation with a dilution of 1:5, agglutination being evident at 1:500 of the unfiltered precipitated mixture. At 3.4 saturation over half and at 3.6 saturation all the agglutinin was precipitated. Again, 20 cc. of the same serum were precipitated with 10 cc. of saturated ammonium sulphate solution; the euglobulin then agglutinated at 1:2400 (in terms of original serum) the pseudoglobulin at 1:20. After two reprecipitations the eu-fraction still reacted at 1:2400.

TABLE VII. FRACTIONING OF POLYAGGLUTINATIVE GOAT SERUM.
Bleeding of III/18/06; fractioned III/20/06.

Organism.	Fraction.	100	200	500	1000
Flexner Manila	Total gbl.	++++	++++	+++	±
	Pseudogbl.	++++	++++	++	−
Shiga	Total gbl.	++++	++++	0	−
	Pseudogbl.	++++	++++	++++	±
Pfeiffer	Total gbl.	++++	+++	±	−
	Pseudogbl.	++++	++++	−	−
Cholera	Total gbl.	++++	++++	+	−
	Pseudogbl.	++++	++++	+	−
Colon	Total gbl.	++++	+++	++	−
	Pseudogbl.	++++	+++	−	−

POLYAGGLUTINATIVE HORSE SERUM.

(*a*) *Antidysentery Horse Serum.* Horse 284; immunized against the Shiga, Flexner Manila and the Mount Desert strains. Bleeding of X/3/06; fractioned X/4/06.

Here (Table VIII) the agglutinins are split between the fractions, the larger part of each occurring in the pseudoglobulin.

(*b*) *Anticholera and Antityphoid Horse Serum.* Horse 254; combined immunization against the original Pfeiffer and cholera. Serum from bleeding on III/27/06; fractioned IV/1/06 and refractioned V/10/06. The results are given in Table IX.

With the cholera-typhoid serum, the agglutination values of which for each organism were in the neighborhood of a dilution

of 1:1000, the bulk of the agglutinins was found in the pseudoglobulin (Table IX); the greater portion was also in the high fraction when the serum was precipitated at 3.6 and again at 3.8 saturation. There is no evidence presented here that the precipitation limits of the cholera agglutinin are in any way different from that of the typhoid. In the second fractionation (V/10/06) it is seen that both the Pfeiffer and the cholera are increased in the eu-fraction as contrasted with the result of the first precipitation at 3.4 saturation.

TABLE VIII. FRACTIONING OF ANTIDYSENTERY HORSE SERUM.

Organism.	Fraction.	50	100	200	500	1000	2000
Flexner Manila....	Serum	++++	++++	++++	++++	+++	++
	Total gbl.	++++	++++	++++	+++	+	+
	Pseudogbl.	++++	++++	+++	+++	+	−
	Eugbl.	++++	++++	+++	±	−	−
Shiga	Serum	++++	++++	++++	++++	+++	−
	Total gbl.	++++	++++	++++	++++	±	−
	Pseudogbl.	++++	++++	++++	−	−	−
	Eugbl.	++++	++++	+++	−	−	−
Colon.....	Serum	++++	++++	++++	++++	++++	0
	Total gbl.	++++	++++	++++	+	−	−
	Pseudobgl.	++++	++++	++++	±	−	−
	Eugbl.	++++	+++	++	±	−	−

The fractionation of the anticholera and antityphoid horse serum is of especial interest because an experiment of this nature is the most striking of E. P. Pick's observations. Pick had found that the typhoid agglutinin was precipitated with the pseudoglobulin fraction in horse serum; the cholera agglutinin, on the contrary, came down with the euglobulin. Pick, therefore, mixed equal volumes of a typhoid and a cholera serum, and progressively precipitated 2 cc. amounts of the mixed sera at 2.8, 3.0, 3.2, etc., saturation (with a final volume of 10 cc. in each case). A well marked separation of the cholera agglutinin into the euglobulin and the typhoid agglutinin into the high fraction resulted. As given in Pick's tables, the observed agglutination values are twice too much, since each serum was diluted a half by the mixing. A direct separation at 3.4 saturation of the agglutinin in a goat cholera serum (agglutinating at a dilution of

TABLE IX. FRACTIONING OF ANTICHOLERA-ANTITYPHOID HORSE SERUM.

1. Precipitation at 3.4 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer.....	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	++++	++++	+++	±
	Eugbl.	+	+	−	−	−
Cholera.....	Total gbl.	++++	++++	++++	++++	++++
	Pseudogbl.	++++	++++	+++	+++	+
	Eugbl.	+	+	−	−	−

2. Precipitation at 3.6 saturation.

Organism.	Fraction.	50	100	00	00	1000
Pfeiffer.....	Total gbl.	++++	++++	++++	++++	++
	Pseudogbl.	++++	++++	++++	+++	−
Cholera.....	Total gbl.	++++	++++	++++	++++	++++
	Pseudogbl.	++++	++++	++++	++++	++++

3. Precipitation at 3.8 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer.....	Total gbl.	++++	++++	++++	++++	±
	Pseudogbl.	++++	++++	+++	+++	±
Cholera	Total gbl. .	++++	++++	++++	++++	++++
	Pseudogbl.	++++	++++	++++	+++	±

4. The same serum was again fractioned on V/10/06.

Organism.	Fraction.	50	100	200	500	1000	2000
Pfeiffer...	Total gbl.	++++	++++	++++	++++		
	Pseudogbl.	++++	++++	++++	+++		
	Eugbl.	++++	++++	++++	−		
Pfeiffer* .	Total gbl.	++++	++++	++++	++++	+++	++
	Pseudogbl.	++++	++++	++++	+++	±	−
	Eugbl.	++++	++++	−	−	−	−
Cholera ..	Total gbl.	++++	++++	++++	++++	+++	
	Pseudogbl.	++++	++++	++++	+++	++	
	Eugbl.	++++	++++	+++	±	−	

* A second fractionation.

1:1000) from the typhoid agglutinin in horse serum (1:20,000) was made. The euglobulin was precipitated by adding a half volume of saturated ammonium sulphate solution directly to the mixed sera. The high agglutination values are again given as *direct* observations, though probably calculated for the original undiluted sera. The figures for the reprecipitated euglobulin are for the typhoid, 1:3000, and for the cholera 1:1600, (an increase over the value of the original goat serum); the pseudoglobulin (3.3 saturation filtrate) agglutinated the typhoid at 1:16,000, the cholera at 1:20.

TABLE X. FRACTIONING OF ANTICHOLERA-ANTITYPHOID HORSE SERUM.
Horse 254; bleedings of II/27/06 and V/29/06; fractioned X/8/06.
Fractions tested with the Mt. Sinai culture of typhoid.

Centrifuged.	Fraction.	100	200	500	1000	2000
	II/27/06					
2 hrs. after precipitation...	Total gbl.	++++	++++	++++	+++	+
	Pseudogbl.	++++	+++	+++	±	
	Eugbl.	++++	+++	+	±	
After 12 hrs.	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	+++	+++	±	
	Eugbl.	++++	+++	+	±	
	V/29/06.					
	Serum	++++	++++	++++	++++	+
After 2 hrs....	Total gbl.	++++	++++	++++	+++	+
	Pseudogbl.	++++	++++	++++	++	±
	Eugbl.	++++	++++	++++	++	±
After 12 hrs....	Total gbl.	++++	++++	++++	++++	+
	Pseudogbl.	++++	+++	+++	+	
	Eugbl.	++++	++	±		

In the following observations it is shown that a relatively large proportion of the typhoid agglutinin may occur in the euglobulin fraction.[1]

Six cc. of each serum were diluted with 13.8 cc. of water and precipitated by the gradual addition of 10.2 cc. of saturated ammonium sulphate solution. Uniform samples (15 cc.) of each

[1] On resuming this problem in the fall of the year, it was found that our cholera culture was spontaneously agglutinating; it could not therefore be employed in testing the agglutination values of the fractions.

were centrifuged after two hours' standing. The euglobulin precipitates were washed by thoroughly suspending the proteid in 15 cc. of 3.4 saturation ammonium sulphate solution and again centrifuging; the precipitates were washed three times in this fashion. Exactly similar precipitations were made on the two sera but the precipitated mixtures were centrifuged after twelve instead of two hours standing. The results are given in Table X.

A high typhoid agglutinin content in the euglobulin was also obtained on fractioning the last bleeding of the cholera-typhoid horse. The results are shown in Table XI.

TABLE XI. FRACTIONING OF ANTICHOLERA-ANTITYPHOID HORSE SERUM.

Horse 254; bled X/3/06; fractioned X/4/06; tested only with typhoid.*

Organism.	Fraction.	100	200	500	1000	2000
Mt. Sinai Typhoid.....	Total gbl.	++++	++++	++++	**++++**	++
	Pseudogbl.	++++	++++	++++	**+++**	−
	Eugbl.	++++	++++	**+++**	−	−
St. Typhoid...	Total gbl.	++++	++++	**++++**	+	+
	Pseudogbl.	++++	++++	**++++**	++	−
	Eugbl.	++++	++++	**+++**	−	−

* The Pfeiffer strain agglutinated spontaneously. The Mount Sinai strain has always shown the same agglutinations as the Pfeiffer in numerous other observations.

FRACTIONATION OF DIPHTHERIA ANTITOXIC GOAT AND HORSE SERUM.

Fresh goat serum and serum from horse 307, VII/5/06, were fractioned as follows: 3 cc. of serum diluted with 6.9 cc. of water were precipitated with 5.1 cc. of saturated ammonium sulphate solution, and the precipitate from a 10 cc. sample of each obtained by centrifuging. The precipitates were suspended in 3 cc. of 3.4 saturated ammonium sulphate solution and again centrifuged; the washing was twice repeated and the wash solutions united and made up to 10 cc. The precipitates were suspended as usual in 3.4 saturated ammonium sulphate and made up to 10 cc. The results calculated per cc. of the original undiluted serum follow:

Fraction.	Goat.	Horse.
Serum	90 units	250 units
Total globulin	90 "	250 "
Pseudoglobulin	35 "	+200 "
Euglobulin	5 "	0* "
Wash solution	50 "	+25 "

* Tested for 5 units against 100 m.l.d.; the guinea pig died in 12 hours, autopsy showing a typical diphtheria toxin picture.

The same results were obtained in two similar experiments. The two sera certainly show a different behavior towards ammonium sulphate precipitation. A relatively large proportion of the goat antitoxin is precipitated with the euglobulin. The facility with which the antitoxin can be washed out almost completely (in a total united volume of wash solution less than the original volume of the precipitated mixture) shows that the antitoxin is not invariably linked to the euglobulin.

Pick's experiment is given briefly for comparison with our results:

Twenty cc. of antitoxic goat serum[1] (neutral reaction) were precipitated with 10 cc. of saturated ammonium sulphate solution. After two hours, the precipitate was pressed out and dissolved in 30 cc. of water; it was reprecipitated at 3.3 saturation and dissolved in 20 cc. of water (euglobulin fraction). The filtrates were united and made up to half saturation, the precipitate dissolved in the original volume (20 cc.) and refractioned between 3.3 and 5.0. The euglobulin then obtained was united with the above euglobulin solution and the mixture precipitated at 3.3 saturation. The serum contained about 10 units per cc. The fractions tested as follows:

---

[1] Pick states (p. 361) "Zu dem nun folgenden Trennungsversuche mit Diphtherie immun Ziegenserum stand mir nur ein Ziegenserum zur Verfügung, von dem 0,1 ccm eben im stande war, die 10 fache tödliche Giftmenge eines Toxins zu paralysieren, das in der Dosis von 0,0098 ccm ein Meerschweinchen von etwa 260 g in drei Tagen tötete." This would make the potency of the serum used only 1 unit per cc. The control test made actually gives the strength as 10 antitoxin units per cc.: 0.098 cc. toxin (10 m.l.d.) were neutralized by 0.01 cc. serum; therefore 100 m.l.d. toxin were neutralized by 0.1 cc., or 1 cc. serum neutralized 10 × 100 m.l.d. toxin. Ledingham has passed this over: "The goat serum with which Pick worked had a very low antitoxic value inasmuch as 0.1 cc. was required to neutralize 10 lethal doses of a toxin whose m.l.d. was only about 0.01 cc." Pick, himself, speaks of the antitoxic value of this serum incidentally "Man erkennt trotz der geringen Wertigkeit des Serums...."

Pseudoglobulin:

0.05 cc. + 10 m.l.d. toxin (testing for 2 units). The guinea pig died on the second day.

Euglobulin:

0.01 cc. + 10 m.l.d. toxin (testing for 10 units). Died on the third day.

0.017 cc. + 10 m.l.d. toxin (testing for 6 units). Induration and loss of weight, but survived.

Ledingham states in his conclusions that in the horse serum the relationship of the diphtheria antitoxin to the pseudoglobulin fraction "holds good only when the antitoxin content of the serum is steadily rising." Horse 307 of this department had been subjected to immunization for over five months; it attained a maximum of over 300 units per cc. in three months and had declined to 250 units two months later when the blood of the serum used in our fractionation experiments was drawn.[1] Apparently Ledingham's conclusion (from observations on a single horse) is not of general application.

Tabulating Pick's results by a somewhat different arrangement than the one presented in his paper (p. 384), it is seen from the following—

Animal.	Diphtheria Antitoxin.	Tetanus Antitoxin.	Cholera Lysin (Pfeiffer.)	Typhoid Agglutinin.	Cholera Agglutinin.
Goat	eugbl.	eugbl.	eugbl.	eugbl.	eugbl.
Rabbit				eugbl.	
Guinea pig..				eugbl.	
Horse	pseudogbl.	pseudogbl.		pseudogbl.	eugbl.

—that there is no evidence of any differences in the precipitation limits of the antibodies in goat, rabbit and guinea pig sera. We should hardly expect *a priori*, then, that a separation of any other antibodies by fractionation of goat and rabbit serum would be possible, and we have not found otherwise. Yet the probability of such a separation for horse serum was suggested by Pick's experiments. With this serum, even, the distribution of the antibodies as determined by Pick, has been similarly homogeneous with the exception of the cholera agglutinin. Pick, accordingly, gives one example, and one only, of an antibody

[1] The horse subsequently was killed as no longer of service for antitoxin production.

differing from other antibodies in the serum of the same species by its precipitation characters toward ammonium sulphate. This observation of Pick's we have been unable to verify when polyagglutinative horse sera have been used. It is probable, however, that the serum globulins of different animals or even of various individuals of the same species may show a different and inconstant behavior quantitatively toward fractional ammonium sulphate precipitation. We have not found that any of the antibodies in goat, rabbit and horse serum were invariably associated with the euglobulin. Our results with goat diphtheria antitoxin have been confirmed by Ledingham. At the same time we have presented repeated observations with several strains of typhoid showing that a large proportion (almost half in some instances) of the typhoid agglutinin of horse serum may be found in the thoroughly washed euglobulin fraction of both old and fresh serum.

The results of our experiments have already been briefly summarized in the preceding portion of the paper.

It is to be hoped that any future work on the fractionation of the antibodies or of the proteids of the blood will not be undertaken without a thorough comprehension of the nature and limitations of the process. Salt fractionation is a valuable method for the purification and preparation of proteid products; the salt concentration precipitation limits, however, are not a reliable means for differently classifying proteids the precipitation characters of which are not widely separated.

THE FRACTIONAL PRECIPITATION OF ANTITOXIC SERUM.

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Comparatively little attention has been paid to the fractional precipitation of antitoxin. Brodie[1] in 1897 separated antitoxin horse serum into four fractions by the progressive addition of ammonium sulphate to half saturation; all four contained, however, relatively equal amounts of antitoxin. Atkinson[2] in this laboratory saturated with sodium chloride a solution of the moist serum globulin precipitate obtained with magnesium sulphate, and by then employing heat differentiated the globulin into several fractions containing antitoxin. The protective properties corresponded roughly to the quantities of serum globulin in the precipitates. In some unpublished experiments he found that alterations of the amounts of coagulated proteid in the several fractions resulted if more magnesium sulphate was added before heating; there were proportionate changes in the distribution of the antitoxin. Owing to the destruction of a portion of the antitoxin at the higher temperature and possible injury by exposing it to heat of less degree, this fractionation must be considered as incomplete and does not exclude a purification of the antitoxin by salt fractionation. The work of E. P. Pick on the ammonium sulphate fractioning of the anti-bodies has been referred to in the preceding communication. Our own experiments have resulted somewhat differently from either those reported by Atkinson or by Pick, and have developed some new and suggestive facts.

On the basis of the solubility of the antitoxic proteids in saturated sodium chloride solution, one of us (Gibson) recently devised a method for the partial purification and concentration

[1] Brodie: *Journ. of Path. and Bact.*, iv, p. 460, 1897.

[2] Atkinson: *Journ. of Exper. Med.*, v, p. 67, 1901.

of antitoxin.[1] This consisted in precipitating the diluted plasma with an equal volume of saturated ammonium sulphate and separating the antitoxic proteids by extracting the precipitate with saturated sodium chloride solution. We now have employed the method of salt fractionation to study further the concentration of antitoxin.

There exists at the present time considerable confusion in comprehending the methods and basic principles of ammonium sulphate fractional precipitation of proteids. The nomenclature which we have employed and which designates the number of cc. of saturated ammonium sulphate solution in 10 cc. of the *precipitated* mixture has been used by some authorities; it avoids the confusion developed by the use of such terms as "per cent $(NH_4)_2SO_4$ solution" and "per cent saturation $(NH_4)_2SO_4$" "per cent of saturated $(NH_4)_2SO_4$ solution" and "per cent saturation $(NH_4)_2SO_4$ solution" and it seems the simplest and best *practical* expression of degrees of saturation yet suggested. We advise that this method be employed in future papers on fractional precipitation.

Mann, in his version of Cohnheim's "*Chemie der Eiweisskörper*," states (p. 292): "As the solubility of ammonium sulphate is 76.8° (per cent ?) at room temperature, it is easy to calculate what percentage of ammonium sulphate is required for bringing about incipient and complete precipitation of any one albumin, as soon as we know what amounts of saturated ammonium sulphate have to be added for any given quantity of fluid." The simplicity of the above method of calculating vanishes when attention is drawn to the fact that while 100 parts of water dissolve 76.8 gms. of dry ammonium sulphate, the volume resulting is increased to 141 cc. so that 100 cc. of the saturated solution actually contain approximately 54 gms. of the salt, and the degree of saturation as indicated by the *content* of dry ammonium sulphate must be calculated with reference to the latter figure. An example will make clearer the above statement: To obtain a concentration of "half saturation" ammonium sulphate, equal volumes of the proteid solution and of saturated ammonium sulphate solution are mixed; according to the apparent meaning of Mann's obscure statement, 100 cc. of the resulting " half saturated" solution would contain 38.4 gms. of the dry salt; it actually does contain 27 gms. of ammonium sulphate.[2]

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[1] The literature on the purification and chemical characters of antibodies has been briefly reviewed in a paper on "The Practical Concentration of Diphtheria Antitoxin for Therapeutic Use," this *Journal*, i, p. 161, 1906, and more recently by Ledingham: *Journ. of Hyg.*, vii, p. 65, 1907.

[2] Because of the change in the volume of the solvent on adding the salt, it is similarly not possible to add 38.4 gms. of ammonium sulphate to 100 cc. of water and have a solution at "half saturation." In this case the volume would be increased to 120.7 cc. and 100 cc. would contain 31.7 gms. of the salt.

E. P. Pick has fallen into the same error in his paper on the fractionation of the anti-substances in the globulins of serum. He speaks (p. 356) of the limits of the various serum fractions as follows: "dass das von Reye aus normalen Pferdeserum abgeschiedene Fibrinoglobulin entsprechend einer Sättigung von 21.5 Proz. Ammonsulfat.... ...ein bestimmter Teil (the euglobulin) des nun übrig bleibenden Globulins keine antitoxische Wirkung hatte und dass sich dieser aus dem Serum noch bequem abschieden liess, wenn die Flussigkeit einer Gehalt von 25.6 Proz. an Ammonsulfat enthielt. Es verblieb nunmehr ein Eiweisskörper in Lösung (the pseudoglobulin) der durch weiteres Eintragen der gesättigten Ammonsulfatlösung bis zu einem Gehalte von 38 Proz. von dem Serumalbumin gut zu trennen ist und den Heilkörper in quantitativer Ausbeute enthält." The precipitation limits are distinctly designated here by 21.5, 26.6 and 38 per cents of ammonium sulphate in the precipitated mixture. They actually mean a content of 2.9, 3.33, and 4.9 cc. of saturated ammonium sulphate solution in 10 cc. of the precipitated mixtures, which would then contain, respectively, 15.67, 18.00 and 26.50 gms. of the dry ammonium sulphate per 100 cc.—figures which by no means or method of interpretation can be logically expressed by 21.5, 25.6 and 38 percentages of ammonium sulphate. Fortunately the fault lies in the nomenclature only, the precipitations being accomplished by the use of saturated ammonium sulphate solution.

Twenty liters of plasma (475 units per cc.) were diluted with 20 liters of water; by fractioning with saturated ammonium sulphate solution, the three proteid precipitates were obtained which separated at concentrations corresponding to 3.3 cc., 3.3–3.8 cc. and 3.8–5.0 cc. of the saturated salt solution in 10 cc. The saturated sodium chloride soluble (antitoxic) globulins of these fractions and of the 5.0 saturation precipitate of a second 20 liters of the plasma were prepared as usual. Proteid determinations (coagulations) and potency tests were duplicated.

Prep. 77.

Fractions.	A. 0.0–5.0	B. 0.0–3.3	C. 3.3–3.8	D. 3.8–5.0
Volume cc.	5200	1440	1400	2050
Units per cc.	1450	1150	1350	1750
Times concentrated	3.05	2.42	2.84	3.68
Per cent recovered	79.3	17.4	19.9	37.8
Proteid, gms. per 100 cc.	11.66	11.51	9.87	9.70
Units, per gm. proteid	12436	10000	13666	18000

A second experiment with a 450 unit plasma gave the following results:

Prep. 82.

Fractions.	A. 0.0–5.0	B. 0.0–3.3	C. 3.3–3.8	D. 3.8–5.0
Volume cc.	6240	1350	1640	2550
Units per cc.	1050	900	1300	1600
Times concentrated	2.34	2.00	2.89	3.56
Per cent recovered	72.8	13.9	22.6	45.3
Proteid, gms. per 100 cc.	10.59	12.06	13.46	13.41
Units, per gm. proteid	9914	7464	9655	11930

These observations show that the antitoxic globulins of the higher fraction are much more potent than those of the less soluble proteids.

Both the preparations by the half-saturation ammonium sulphate method and by fractioning, when precipitated from the saturated sodium chloride solution and dialyzed, contained a probably partially denaturalized antitoxic globulin; this had a diminished solubility and antitoxic potency (per gram proteid) and was precipitated on slight acidification by diluting twenty times. The filtrates from the water-acid precipitable globulin coagulated at 73°, while saline solutions of the precipitates so obtained showed varying and much lower coagulating temperatures. The solutions of the high proteid fractions have a peculiar green color. A redetermination of the precipitation limits of the globulin in the three fractions after removal of the water-acid precipitable proteid, showed that the different precipitation limits were relatively characteristic for the fractions.

The following results were obtained on progressively fractioning (in two experiments) by the addition of the dry salt[1] to a

[1] Calculations or reference tables for the amounts of salt to be added to produce or raise a proteid solution to any desired concentration may accurately be made by employing the following formula:

$$X = \frac{v\,p\,(c_2 - c_1)}{10 - e\,p\,c_2}$$ where x is the number of gms. of salt to be added to give the required concentration, v the original volume in cc., e the increase in the volume of the solvent by 1 gm. of salt, p the gms. of salt per cc. of its saturated solution, and c_1 and c_2 are the initial and desired degrees of saturation, expressed as cc. in 10 cc. For $(NH_4)_2SO_4$ e and p may be regarded as approximately 0.54; then

$$X = \frac{v\,(c_2 - c_1)}{18.158 - 0.54\,c_2}; \text{ and when } c_1 = 0,\ X = \frac{v\,c_2}{18.158 - 0.54\,c_2}$$

To raise the concentration by the addition of saturated salt solution the

liter of about 400 units antitoxic plasma. The initial dilution was 1:5. The precipitates were pressed between filters and extracted with saturated sodium chloride solution. The determinations on the filtered extracts are given per cc. of the original plasma. The results are roughly quantitative only, loss of the filtrate in pressing out the precipitated globulins being disregarded. Proteid determinations and potency tests were duplicated.

FRACTIONING OF PLASMA 305, 8/1/06.

Fractions.	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
A			
0.0–3.4	0.00321	25	7788
3.4–3.6	0.00223	20	8968
3.6–3.8	0.00432	47	10879
3.8–4.0	0.00416	52	12500
4.0–4.2	0.00408	60	14705
4.2–4.4	0.00272	55	20220
4.4–4.6	0.00191	40	20942
4.6–4.8	0.00163	32	19632
4.8–5.0	0.00111	19	17117
5.0–5.6	0.00428	18	4205
B			
0.0–3.4	0.00394	27	6853
3.4–3.6	0.00219	20	9132
3.6–3.8	0.00397	45	11335
3.8–4.0	0.00336	50	14880
4.0–4.2	0.00332	60	18072
4.2–4.4	0.00255	55	21568
4.4–4.6	0.00181	40	22094
4.6–4.8	0.00147	30	20408
4.8–5.0	0.00093	18	19355
5.0–5.6	.....	18	.....

In each instance there is a progressive increase in potency as the antitoxic globulin becomes more soluble in the fractions until a concentration of the salt of about 4.2 is reached. The potency per gram remains then practically constant at about three times that of the saturated sodium chloride extract of the euglobulin fraction (0.0–3.4) until between 4.8 and 5.0 saturation; above this limit the potency per gram rapidly diminishes to a relatively very low figure. Between the 4.2 and 4.8 limits, over

amounts (cc.) of the original proteid solution and of the saturated salt solution in the mixture are calculated; also the amount of the salt solution necessary to bring the *proteid solution* to the desired concentration. Sufficient excess of saturated salt solution over that already present is added to make the required total.

half the units of the original plasma are precipitated, while the antitoxin is contained in less than one-third of the total antitoxic globulin.

The fact that the major portion of the antitoxin remained soluble at a concentration of 4.2 saturation, led us to investigate whether the protective material was mechanically precipitated with the proteid of the lower fractions. Such a result seemed *a priori* improbable because the individual fractions were at such frequent intervals and contained such a small amount of the globulin precipitate as to make hardly conceivable a mechanical inclusion of more soluble colloidal particles for more than a few minutes' duration. Our plan was to fraction the antitoxic plasma at 4.2 saturation. The lower fraction (precipitate) was to be dissolved in an added known volume of water, reprecipitated at 4.2 saturation and after standing 24–48 hours was to be filtered. The procedure was twice repeated with the precipitates obtained at 4.2 saturation. The three filtrates and the saturated sodium chloride soluble (antitoxic) globulin of the final 4.2 saturation precipitate were examined for globulin and antitoxic content. We were not able to separate by three times repeated fractioning at 4.2, the antitoxin from the lower fraction; over half the antitoxin brought down at first was pronouncedly a constituent of the precipitate, the amount in the filtrate from the final precipitation being very slight (though the potency per gram of proteid was relatively high). The protocol follows:

250 cc. of antitoxic plasma (305, 8/10/06, 300 + units per cc.) were diluted with 475 cc. of water and precipitated with 525 cc. of saturated ammonium sulphate solution. After standing 24 hours, the precipitated globulin was filtered off. To the filtrate, 1000 cc., was added 60 gms. of dry ammonium sulphate after sufficient ammonium sulphate solution had been employed to give 1500 cc. at half saturation. The resulting precipitate (4.2–5.5 saturation) was pressed out between filters, dissolved and made up to 200 cc.

The precipitate at 4.2 saturation was pressed out between filter paper, dissolved by the addition of 580 cc. of water and reprecipitated with 420 cc. of saturated ammonium sulphate solution. The total volume of the precipitated mixture was slightly over 1000 cc. After standing 24 hours, the reprecipitated globulin was filtered off from "Filtrate I" (900 cc.).

The precipitate from I was dissolved in 580 cc. of water and precipitated with 420 cc. of saturated ammonium sulphate. Filtrate II was 930 cc.

Filtrate III similarly obtained amounted to 950 cc. The globulin precipitate was extracted with 1000 cc. of saturated NaCl solution.

Determinations of proteid and antitoxic content are given per cc. of the original plasma.

	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
Filtrate I.....	0.00270	40	14810
" II.....	0.00332	50	15080
" III.....	0.00075	12	16100
4.2 Ppt. gbl. (ext. NaCl)	0.01066	125	12100
4.2–5.6.	0.00685	80	11680
	0.02428	307	

Further fractioning after complete removal of the water precipitable globulin was done on 50 cc. of the globulin solution, Prep. 77 A (cf. p. 255). The fractioning was made at a dilution of the original preparation of 1:20. The results are expressed per cc. of the original undiluted preparation.

REFRACTIONATION OF PREPARATION 77A.

Fraction.	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
0.0–4.0	0.0408	400	9791
4.0–4.4	0.0165	225	13667
4.4–5.0	0.0176	375	21306
5.0+	0.0018	75	41722
4.8–5.5*	0.0046	150	34783

* Made on a second 50 cc. of the same preparation.

The refractioning of 77A from which the water-acid precipitable globulin had been removed, showed a marked progressive increase in potency hand in hand with the greater solubility of the proteid.

Fraction 3.8–5.0 of Prep. 82 was refractioned without removing the water-acid precipitable globulin. The dilution was 1:10.

REFRACTIONATION OF PREPARATION 82D. (High Potency Fraction.)

Fraction.	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
0.0–4.0	0.07318	600	8136
4.0–4.2	0.01779	240	13490
4.2–4.4	0.02197	260	11840
4.4–4.6	0.01232	160	12990
4.6–4.8	0.00708	90	12711
4.8–5.0	0.00511	80	15670
5.0–5.6	0.00197	90	45690
	0.13941	1510	
For 82D.	0.1341	1600	

82 D contained a globulin of rather uniform potency per gram from fractions 4.0–4.8; then a marked jump for the fraction 5.0–5.6 to about three times the original potency per gram was observed. The portion of antitoxin in the highest fractions of 77A and 82D was less than 6 per cent of the total units. Prepared for administration as is the ordinary antitoxic globulin, the resulting product would have had a potency of from 5000–6000 units per cc.

The high antitoxic potency per gram proteid of the globulin in the preparations precipitable between 4.8 and 5.6 saturation led us to attempt to obtain such a product in bulk from the antitoxic globulin preparations (Gibson) and directly from plasma. Our experiments have comprised (1) the influence of the reaction of the plasma, (2) repeated extraction (with 4.8 saturation ammonium sulphate) of the globulins to dissolve out the mechanically precipitated highly potent antitoxic substances, and finally (3) progressive denaturalization of the globulin by repeated extractions with saturated sodium chloride solution and reprecipitation with the sulphate. Our results, however, have not been encouraging. The protocols are given below:

(1) *a.* 250 cc. of antitoxic plasma (262, 10/22/06, 500 units per cc.), were diluted with 1050 cc. distilled water and precipitated at 4.8 with 1200 cc. of saturated ammonium sulphate solution. After standing three hours it was filtered and the filtrate (2250 cc.) raised to 5.0 saturation by adding 28.6 gms. of dry $(NH_4)_2SO_4$. After 24 hours at room temperature, the half saturation precipitate was filtered off, pressed between filters and made up to 225 cc. Of the 5.0 saturation filtrate, 2200 cc. were precipitated at 5.6 saturation with 96 gms. of dry $(NH_4)_2SO_4$ and filtered after 24 hours' standing at room temperature. The precipitate was pressed out and made up to 218.5 cc. in distilled water.

b. 250 cc. of the same plasma were made distinctly alkaline with $\frac{N}{10}$ NaOH and then fractioned exactly as in *a*.

c. 250 cc. of the plasma were made distinctly acid with dilute acetic acid, and then similarly fractioned.

Proteid coagulations and potency tests were made as usual.

Plasma	Fractions	Proteid per cc.	Units per cc.	Units per Grams of Proteid.
a. Native.....	4.8–5.0	0.0015	11	7333
	5.0–5.6	0.0051	20	3921
b. Alkaline....	4.8–5.0	0.0011	11	10000
	5.0–5.6	0.0047	20	4255
c. Acid.....	4.8–5.0	0.0012	11	9175
	5.0–5.6	0.0060	24	4000

(2) 500 cc. of the plasma of the bleeding employed for reprecipitation at 4.2 (305, 8/10/06, 300 units per cc.)[1] were diluted with 800 cc. of water and precipitated at 4.8 saturation with 1200 cc. of saturated ammonium sulphate solution. After standing for 24 hours, the precipitate was filtered off. Of the filtrate, 2250 cc. were precipitated, at about 5.6 saturation, with 116 gms. of dry $(NH_4)_2SO_4$; after standing, the precipitate was separated, pressed out between filter paper and made up in solution to 450 cc. with water (fraction 4.8–5.6). The moist precipitate obtained at 4.8 saturation was thoroughly suspended in about 1500 cc. of 4.8 saturation $(NH_4)_2SO_4$ and filtered after standing for two days, during which time the mixture was occasionally shaken up. The precipitate from the first filtrate (I) was reëxtracted as before, this procedure being carried on, in all, four times. The precipitate then remaining was made up with saturated NaCl solution to a volume of 1000 cc. Proteid determinations and the antitoxin tests were made on the fraction 4.8–5.6, on the four filtrates and on the NaCl extract of the residue, and are tabulated as before per cc. of the original plasma.

	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
4.8–5.6 sat.	0.00191	12*	6316
Filtrate I.	0.00240	10*	4170
" II.	0.00160	9*	15000
" III.	0.00026	8*	30770
" IV.	0.00030	4*	13333
Residue	0.01784	250	14020
	0.02431	293	

* Because of the low antitoxic and high (toxic) $(NH_4)_2SO_4$ content, the tests were made with 25 or 50 m.l.d. instead of the 100 m.l.d. ordinarily employed.

The amount of antitoxin and of proteid in the filtrates was so small that slight errors in the determinations would influence greatly the calculations of the antitoxin units per gram of proteid. Yet the results obtained on Filtrate III, when the figures on the preparations are recalled (p. 259), make it highly probable that a very small portion of the antitoxin can be separated in a much more highly potent form than is the case for the bulk of this substance.

Progressive denaturalization of the proteid as a means of separating the globulin from the antitoxic substance, if other than the serum globulin itself, has not proved successful. The method used was to extract the ammonium sulphate globulin precipitates of plasma or the antitoxic globulin preparation with saturated

[1] Cf. pp. 258 and 259.

sodium chloride solution, to filter off the insoluble globulin residue (after some days standing), and to reprecipitate the filtrate by the addition of a little over half its volume of saturated ammonium sulphate solution. The extraction of this last precipitate with the sodium chloride and the precipitation of the filtrate with ammonium sulphate followed. This procedure was carried on 4–6 times on the 4.8 residue of (2), and on two antitoxic globulin solutions, which were obtained by the sodium chloride extraction of 3.8 saturation precipitates (less potent fraction), one of which was already thoroughly denaturalized in preparation because of the accidental partial desiccation of the acidified saturated sodium chloride precipitated proteid before dialysis. The final filtrates of one of the sodium chloride extracts of the originally denaturalized antitoxic globulin preparations had a potency of almost 15,000 units per gram of proteid. The globulin solution of the 4.8 saturation extraction precipitate and the second antitoxic globulin preparation contained about 10,000 units per gram of proteid.

The injection of the antitoxic globulins of the various globulin preparations sensitizes guinea pigs to subsequent, otherwise nonfatal intraperitoneal administration of serum (Smith and Rosenau and Anderson). Injected intraperitoneally into sensitized guinea pigs, the typical convulsions produced by serum are incited, and the deaths of the animals may ensue. Rashes of the urticarial character with little or no accompanying constitutional symptoms may follow the therapeutic administration of the several fractionally precipitated antitoxic globulins. Therapeutically there is no difference in the results obtained with the equivalent unit injections of either the high (3.8 + saturation) or low (3.3 saturation) fractions of preparations 77 and 82 (pp. 255 and 256).

CONCLUSION.

From the data presented, it appears that the *saturated sodium chloride soluble* serum globulins of the higher fractions are uniformly much more potent per gram of proteid in antitoxin than are those precipitated by lower concentrations of ammonium sulphate. Between concentrations of the sulphate of 5.0 and 5.6, a small proportion of the total sodium chloride soluble glob-

ulin of the antitoxic globulin preparation (Gibson) or of a higher fraction of the same is precipitated; the solution of this globulin has a protective power of over 40,000 units per gram of proteid. The direct fractioning of the plasma, however, does not yield so potent a product; at a dilution of 1:5 of a 400 unit plasma the globulin remaining in solution at 4.2 and precipitated at 4.8 saturation has a potency of about 20,000 units per gram of proteid. It is thus practicable to prepare an antitoxic solution of over 2000 units per cc. from a relatively low plasma.

Whether or not this difference in the potency per gram of proteid is associated with the presence of non-antitoxic globulins having the same fractional precipitation limits as the protective substance remains as yet undecided. It is possible that such a variation in potency may be purely physical, associated with the size or condition of aggregation of the colloidal globulin particles—the less soluble larger masses having diminished antitoxic properties. Certainly, however, we find the antitoxin is characterized by a wide range of the precipitation limits similar to the soluble globulins, *i. e.*, in spite of repeated precipitations, a part of the antitoxin is comparatively insoluble in concentrations of ammonium sulphate in which the major portion of the protective substance readily dissolves.

In concluding the present paper, we desire to express our appreciation of Dr. Park's suggestions and helpful criticism.

A STUDY OF THE PROTEOLYTIC CHANGES OCCURRING IN THE LIMA BEAN DURING GERMINATION.

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(*I. Contribution from the Agricultural Chemical Laboratory of the University of Wisconsin.*)

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Assuming that during germination catabolic processes predominate in the cotyledon and anabolic processes are most active in the growing stem, then there must be a great difference in the nature of the various nitrogenous bodies of the seed, cotyledon and stem at different stages of development.

In view of the interest and importance from a physiological and chemical standpoint, of a better knowledge concerning these proteolytic changes, the following investigation was undertaken.

As a very satisfactory available material, the lima bean (*Phaseolus lunatus*) was chosen for this study. Beans of about the same size and in perfect ripeness were selected in order to obtain as homogeneous a sample as possible. A part of the seeds was ground and analyzed by the method detailed below and another part placed in sand, kept moist and allowed to germinate at a suitable temperature in darkness. After six days of growth the etiolated plants were 8–12 centimeters long and had a single pair of small leaves 1–1.5 centimeters in length. The temperature of germination, 90° F., seemed to have been favorable for rapid growth, for thereafter the other samples did not grow so fast. From these plants only perfectly normal specimens were selected for analysis, while a part of the remainder was placed again in darkness and the other part left to grow in sunlight. After six days more of growth the plants were harvested as before. They were now 25–30 centimeters long and had two pairs of leaves. The green plants looked larger than the etiolated ones, but the latter were not so perfectly etiolated as expected, the covering having been imperfect, showing a light, pale color.

The difference in color and growth of the two samples, however, was marked. The cotyledons were separated carefully from their leaf-bearing stems.

The fresh samples of six-days-old plants were ground in a mortar and extracted with cold water. The twelve-days-old plants were dried at from 50–60° C. after the cotyledons had been separated. During drying[1] at from 60–70° C. a slight loss of nitrogen may have occurred. Schulze also found that after drying the ammonia content showed a higher value, probably due to a hydrolyzing of acid amide.

From our several materials we then had the following samples for analysis:

I.	Seed.						
II.	Cotyledon	of	6-days-old	etiolated	plant.		
III.	Stem	"	"	"	"	"	
IV.	Cotyledon	of	12-days-old	etiolated	plant.		
V.	Stem	"	"	"	"	"	
VI.	Cotyledon	"	"	"	"	green	plant.
VII.	Stem	"	"	"	"	"	"

METHOD OF ANALYSIS.

All nitrogen determinations were made by the Kjeldahl method.

A. Total nitrogen of substances.

B. Nitrogen of water extracts of substance. (100 parts of cold water were added to one part of sample and after frequent shaking for six or seven hours filtered.)

(1) *The Total Nitrogen of the Water Extract.* An aliquot part of the water extract was taken for the estimation of nitrogen.

(2) *The Nitrogen Insoluble in Water* was calculated from the result of *A* and (1).

(3). *Nitrogen of Total Proteids.* Of the various methods for estimating proteids, Stutzer's has been most commonly used.[2] Since cupric hydroxide forms crystallizable, insoluble substances with certain amino-acids, it is possible that under the conditions of proteolysis some amino-acids may result and may be thrown down along with proteids, as shown by Scherning's work;[3] it is doubtful, however, if this would be a disturbing factor in the analysis of natural grains, fodders, etc. The chief objection to

[1] E. Schulze: *Zeitschr. f. physiol. Chem.* xxiv, pp. 42, 43, 1898.

[2] *Journ. f. Landw.*, p. 103, 1880.

[3] *Zeitschr. f. anal. Chem.*, xxxix, p. 545, 1900.

Stutzer's method for this work is that the precipitation of albumoses and peptones by cupric hydroxide is not complete. Stutzer[1] states that cupric hydroxide completely precipitates albumoses but only partly precipitates peptones; yet in the author's experiments with the seeds of the lima bean it appeared to throw down even albumoses incompletely, giving a slightly lower result than the method in which a saturated solution of zinc sulphate was employed. N. Nedokutschajen[2] also obtained a low result with Stutzer's method in an analysis of certain grains and Frankfurth[3] has called attention to the incomplete precipitation of albumoses by cupric hydroxide.

These results do not, however, lead to the conclusion that cupric hydroxide does not precipitate completely all albumoses. We are led therefore to consider whether Stutzer's reagent does or does not completely precipitate albumoses of some kinds and only partially throws down or does not at all precipitate some other kinds; as, for example, it has been shown that copper sulphate or copper acetate[4] precipitates primary albumoses (hetero- and proto-) but does not precipitate deutero-albumoses.

In an experiment with a mixture of Witte's peptone and the acid decomposition products of casein, Stutzer's method gave a higher value than that with zinc sulphate. This mixture contained:

In 100 cc.	.012 gm. N	as albumoses.
	.031 " "	" peptones.
	.033 " "	" amino-bodies.
In 100 cc.	.015 gm. N	by Stutzer's method.
	.012 " "	" zinc sulphate method.

To explain this result, it must be proved whether or not cupric hydroxide precipitated part of the albumoses and part of the peptones and their sum exceeds the albumoses precipitated by zinc sulphate, or completely precipitated albumoses and partly precipitated peptones, as anticipated from the above statement of Stutzer. We must also consider what influence the presence of amido-bodies may have had upon the yield of nitrogen. The

[1] *Zeitschr. f. anal. Chem.*, xxxi, p. 505, 1892.

[2] *Landw. Versuchsstat.*, lviii, p. 273, 1903.

[3] *Ibid.*, xlvii, p. 451, 1895.

[4] Hoppe-Seyler: *Handbuch d. chem. Anal.*, p. 324. Folin: *Zeitschr. f. physiol. Chem.*, xxv, p. 152, 1898.

above result suggests the question: Do the albumoses in seeds have a close relation to deutero-albumoses?

At least, we can say that cupric hydroxide does not perfectly precipitate the albumoses of the lima bean and of some other seeds, and consequently Stutzer's method is not suitable for the analysis of some plant seeds and young plants containing albumoses.

The tannin salt method[1] proposed for the analysis of meat extracts gave more satisfactory results in the hands of the author than the cupric hydroxide methods.

(4) *Nitrogen of Coagulable Proteids.* The water extract was boiled for a few minutes with the addition of one or two drops of acetic acid, and treated in the usual way.

(5). *Nitrogen of Ammonia.* For the determination of ammoniacal nitrogen the filtrate from (4) was distilled with magnesium hydroxide at 38–40° C. under reduced pressure. Ammonia in the water extract of plants has commonly been determined according to the method of E. Bosshard[2] with the use of phosphotungstic acid. Bosshard obtained ammonia quantitatively from ammonium chloride solution with the above reagent. In preliminary tests by the author on ammonium sulphate solution, phosphotungstic acid gave less accurate results, as shown by the following table:

N per 100 cc. of $(NH_4)_2SO_4$ solution (in gram).	N precipitated from $(NH_4)_2SO_4$ solution by phosphotungstic acid. (Per cent of total nitrogen.)
.0299	52.1
.0150	52.1
.0093	53.4
.0047	73.0
.0024	76.8
.0012	60.6

These results led to the rejection of Bosshard's method for our purpose.

The question whether acid amide compounds, such as asparagin and glutamin, lose their amide group by cleavage in heating the solution with magnesium hydroxide has been conclusively answered in an article by E. Schulze.[3] In digestion with mag-

[1] *Journ. of the Amer. Chem. Soc.*, p. 1497, 1906.
[2] *Zeitschr. f. anal. Chem.*, xxii, p. 329.
[3] *Landw. Versuchsstat.*, lxv, p. 237, 1906.

nesium hydroxide at boiling temperature under ordinary atmospheric pressure, a part of the nitrogen splits off, but no cleavage occurs in vacuum distillation at 40° C.

(6) *Nitrogen of Albumoses;* the solution from the determination of ammoniacal nitrogen was treated with zinc sulphate as substituted by Bömer[1] for ammonium sulphate. He suggests a correction of the results obtained because of the probable formation of double salts of zinc sulphate with ammonium sulphate. In using this method on the mixtures of albumoses, peptones and amid-bodies described under (3), containing ammonium sulphate equal to 0.024 gram nitrogen per 100 cc., precipitation of ammonium salts did not occur, as proved by distilling with magnesium hydroxide. The only difficulty with this method was the slowness of filtration but this was modified so that one worked with a definite volume of saturated zinc sulphate solution and estimated the nitrogen in an aliquot portion of the filtrate. According to the work of Pinkus, Shafer and Haslam[2] sodium sulphate has come into use as a substitute for zinc sulphate in the separation of albumoses. In experimenting with a saturated solution of sodium sulphate, after the manner of the zinc sulphate method, keeping the solution at 36–39° C.—above the transition point of the two systems of sodium sulphate salts, clear separation and rapid filtration was obtained. The following results indicate a close agreement with the zinc sulphate method.

$ZnSO_4$ gave 0.284 per cent N of albumoses.
Na_2SO_4 " 0.276 " " " "

(7) *The Nitrogen of Peptones* was calculated by subtracting the sum of the nitrogen of coagulable proteids and albumoses from the nitrogen of total proteids found by the tannin salt method.

(8) *Nitrogen of Diamino-Compounds;* the solution from the determination of ammoniacal nitrogen was submitted to Hausmann's method for the separation of diamino-compounds from monoamino-compounds.[3] In our tests the freshly prepared reagent was believed to have a higher precipitating value than

[1] *Zeitschr. f. anal. Chem.*, xxxiv, p. 562, 1895.

[2] G. Mann: *Chemistry of Proteids*, p. 185.

[3] Discussion and criticism of this method will be found in Cohnheim's *Chemie der Eiweisskörper*.

older reagents and we thought it might be difficult to obtain accurate results by the method; it was thought suitable, however, for comparative work on diamino-compounds. The work was carried out in accordance with the method proposed for the analysis of cheese and milk by VanSlyke and Hart.[1]

(9) *Nitrogen of Monoamino-Compounds;* the total nitrogen in the filtrate from the above phosphotungstic acid precipitate was estimated as the nitrogen of monoamino-compounds.

(10) *Nitrogen of Acid Amides;* proteids were separated from the water extract of the samples by lead acetate, the filtrate freed from lead by hydrogen sulphide, the solution then digested with dilute acid, neutralized with alkali and distilled with magnesium hydroxide for ammonia. Subtracting from this result the value obtained for ammonia under (5) we have the equivalent of nitrogen in acid-amide combinations.

TABLE I.

NITROGEN EXPRESSED AS PERCENTAGE OF DRY MATTER.

Name of Nitrogen.	Seed	Cotyledon.			Stem.		
		6-days etiol.	12 days Plant		6-days etiol.	12-days Plant	
			etiol.	green		etiol.	green
N total	3.023	2.751	2.908	2.845	5.116	5.736	5.081
N insoluble in water	0.698	0.725	0.979	1.193	0.177	1.363	1.917
N coagulable	1.711	0.701	0.677	0.543	trace	0.314	0.358
N albumoses	0.281	0.205	0.145	0.148	0.056	0.082	0.017
N peptones	0.005	0.021	0.032	0.005	0.309	0.481	trace
N diamino-compounds	0.023	0.047	0.024	trace	0.017	trace	trace
N monoamino-compounds	0.307	0.920	0.985	0.899	4.265	3.330	2.698
N ammonia	0.007	0.132	0.066	0.057	0.292	0.166	0.093
N acid amides	0.016	0.112			0.652		

TABLE II.

THE NITROGEN EXPRESSED AS PERCENTAGE OF TOTAL NITROGEN.

N total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
N insoluble in water	22.79	25.99	33.66	41.93	15.91	23.76	37.72
N coagulable	56.59	25.52	23.28	19.08	trace	5.47	7.04
N albumoses	9.29	7.45	4.98	5.20	0.95	1.42	0.33
N peptones	0.16	0.93	1.10	0.17	5.14	8.38	trace
N diamino-compounds	0.76	1.51	0.82	trace	0.28	trace	trace
N monamino-compounds	10.15	33.79	33.87	31.59	72.85	58.05	53.09
N ammonia	0.23	4.77	2.26	2.00	4.85	2.89	1.83
N acid amides	0.52	4.07			11.14		

[1] *Amer. Chem. Journ.*, xxix, 1903.

TABLE III.

GRAMS OF NITROGEN IN PLANTS FROM 1000 SEEDS.

	Seed	Cotyledon.			Stem.		
		6-days etiol.	12-days etiol.	Plant green	6-days etiol.	12-days etiol.	Plant green
N total	28.50	16.58	7.56	6.28	5.93	16.36	18.08
N insoluble in water	6.49	4.37	2.54	2.63	0.20	3.95	6.82
N coagulable	16.13	4.22	1.76	1.20	trace	0.91	1.27
N albumoses	2.64	1.23	0.37	0.32	0.06	0.23	0.06
N peptones	0.04	0.12	0.08	0.01	0.35	1.39	trace
N diamino-compounds	0.21	0.28	0.06	trace	0.02	trace	trace
N monoamino-compounds	2.89	5.54	2.56	1.98	4.94	9.65	9.60
N ammonia	0.06	0.79	0.17	0.12	0.33	0.48	0.33
N acid amide	0.15	0.67					

EXPLANATION OF TABLES.

I. Cotyledons.

(1) Total nitrogen. The values of the nitrogen of dry matter are not greatly different but the figures for nitrogen of 1000 seeds and of 1000 cotyledons decrease with the growth of the plants, simultaneously with a decrease of dry matter in the cotyledon and an increase of that in the stem. The following table shows the decrease of dry matter and nitrogen of the cotyledons.

	Seed.	6-days plant. Cotyledon.	12-days etiolated plant. Cotyledon.	12-days green plant. Cotyledon.
Per cent dry matter of seed	100.0	75.4	32.5	27.6
N per cent of total nitrogen	100.0	58.1	26.5	22.0
Ratio of dry matter to nitrogen	1 : 1	1.20 : 1	1.22 : 1	1.26 : 1

Growth continues with about the same ratio of dry matter to nitrogen in the cotyledon of the etiolated plant, but in the cotyledon of the green plant, this ratio increases showing that a comparatively larger amount of non-nitrogenous substances exists in the cotyledon while the nitrogen passes into the stem in larger amounts than in the case of etiolated plants. This emphasizes an old truth that the synthesis of non-nitrogenous compounds is more active in light than in darkness.

(2) The nitrogen insoluble in water may be mostly that of insoluble proteids. While the figures for the percentage of this nitrogen to dry matter (Table I) and to total nitrogen (Table II) show an increase, this increase does not represent an increase of actual weight of nitrogen in the cotyledon. In Table III the quantities of nitrogen of 1000 seeds and of 1000 cotyledons decrease gradually from 6.49 grams in the seed to 4.37 grams in 6-days etiolated cotyledon; to 2.54 grams in 12-days etiolated cotyledon; and to 2.63 grams in 12-days green cotyledon. The following table shows the total weight of nitrogen of insoluble proteids in 1000 plants (cotyledon and stem):

Seed	6.49 grams
6-days old etiolated plant	4.57 "
12-days old etiolated plant	6.49 "
12-days old green plant	9.45 "

From the results of this table it appears that, after decomposition, part of the insoluble proteids passes into the stem and again rebuilds insoluble proteids. The fact that 9.45 grams of nitrogen as insoluble proteids in the green plant is greater than that of the seed proves that forms of nitrogen other than that of insoluble proteids have entered into the formation of the insoluble proteids in the stem. Briefly, the water-insoluble proteids enter into physiological functions in germination, though it is not known with certainty in what form they pass into the stem.

In view of the fact that there exist in the plant proteids insoluble in water, there is no justification for the opinion that the water-insoluble portion cannot pass from cell to cell through the cellulose wall or phloem. We must remember that plant sap is a dilute salt solution, a medium in which at least globulins and even certain albumins are to some extent soluble.

(3) Nitrogen of coagulable proteids. This group contains the greater part of the proteids of the albumin-group and possibly some globulins, since the presence of soluble salts in the plant can influence the composition of the water extract. The decrease of these coagulable proteids, as shown by Tables I, II and III, is very conspicuous. Apparently the proteids of this class play a prominent part in the catabolic processes of the cotyledon. The following table gives the total nitrogen of coagulable proteids in 1000 seeds, and 1000 plants (cotyledon and stem):

Seed	16.13 grams
6-days old etiolated plant	4.22 "
12-days old etiolated plant	2.67 "
12-days old green plant	2.47 "

From a study of this table it seems probable that the coagulable proteids decompose in the cotyledon and pass into the stem to again form protein substances; it would seem improbable, from general physiological considerations, that the coagulable proteids pass into the stem and there suffer proteolysis.

Osborne[1] states that an albumin contained in the wheat kernel differs from animal albumin in being precipitated by saturating its solution with sodium chloride or magnesium sulphate. In this connection it was thought that it would be important to make a similar observation on the lima bean. This test applied to our work, gave the following results:

	Water Extract.	Dilute NaCl Extract.
Nitrogen of coagulable proteids 1 per cent dry matter	1.711	1.830
Nitrogen of proteids salted out by $MgSO_4$		1.251

If 0.119 (1.830–1.711) is the value of globulin, 1.132 (1.251–0.119) should be the value for nitrogen of the proteids other than globulin, such as albumins, which correspond to the proteids found by Osborne. The above result is in harmony with that of Osborne on the wheat in that the albumins of the lima bean are precipitated by saturation with magnesium sulphate.

(4) Nitrogen of albumoses. The decrease of the easily soluble albumoses in the cotyledon raises the question: Do the albumoses pass into the stem without further cleavage and form higher (more complex) proteids? This question cannot be decided from the results of the previous tables, but the figures for albumoses of the cotyledons, as well as those for insoluble and for coagulable proteids, decrease noticeably at the 6-day and 12-day stages.

(5) Nitrogen of peptones, diamino-compounds, mono-amino-compounds and ammonia. Peptones have increased at the 6-day stage and the 12-day stage shows a decrease. The small amount of peptones in the cotyledons of the green plants at the

[1] *Amer. Chem. Journ.*, xv, No. 6, p. 77.

latter stage is especially noticeable, while the green stems show only a trace of these bodies. It seems reasonable to believe that this dearth of peptones is the result of active chemical transition.

From Table III it appears that the fluctuations of peptones content follow those of diamino-compounds.

The decrease of monoamino-compounds and ammonia at the 12-day stage might result from translocation to the stem.

II. The Stem.

(1) Total Nitrogen. The nitrogen of the dry matter has increased at the 12-days stage of etiolated stems while the 12-days-old green stems have a little lower percentage of nitrogen, a relation which illustrates a well known fact that the formation of dry matter is more rapid with than without sunlight. The actual weights of total nitrogen and of dry matter of the stem gradually increase, the following table showing their ratio:

	Stem 6-days plant.	Stem 12-days etiolated plant.	Stem 12-days green plant.
Dry matter in 100 parts (stem of 6-days plant)	100	250	307
Nitrogen per cent of total nitrogen in stem of 6-days plant	100	280	304

(2) Nitrogen of insoluble proteids. We have observed a conspicuous decrease in the actual weights of coagulable proteids of the cotyledon and now we find that the insoluble proteids show a great increase in the stems of etiolated and especially of green plants. This is a most interesting result and suggests the possibility that the "fixed" or non-diffusible proteids play an important part in the formation of the growing stem.

(3) The nitrogen of coagulable proteids increases in value like that of the insoluble proteids though not so much. At the 6-days stage coagulable proteids were not found in the water-extract on boiling, nor on adding acetic acid in the presence or absence of sodium chloride. This fact had already been observed. S. Frankfurth's investigation of the wheat embryo[1] shows the absence of coagulable proteids in the water extract but shows the presence of albumoses. The author, thinking there might be a

[1] *Landw. Versuchsstat.*, xlvii, p. 453, 1895.

stage of growth at which the coagulable proteids disappear through cleavage or the formation of new substances, endeavored to find such a stage in the development of lima and navy beans; but plants of rapid growth, attaining a height of 8–12 centimeters in 6 days, were not obtained and the water extracts of the plant of slow growth showed at each day of growth the presence of proteids coagulable on boiling with acetic acid.

(4) Nitrogen of albumoses. From Table III we see that the albumoses have increased at the 12-days stage in darkness but have decreased in the sunlight. It seems probable that the albumoses took part in the formation of higher proteids in the stem of the green plant. This supposition is supported by the finding of traces of peptones and diamino-compounds in the green stem and by the slightly further decrease of monoamino-compounds and ammonia in the green stem as compared with the etiolated stem at the above stage while at the same stage the higher proteids show greater increase in the stems of green plants.

(5) Nitrogen of peptones. Peptones have increased during 12-days growth in darkness and for the same time have decreased in the sunlight. While the fluctuations of albumoses in the stem resemble those of peptones, in the cotyledon, the value for peptones resemble those for diamino-compounds.

(6) The nitrogen of diamino-compounds occurs only in traces with both etiolated and green plants. It is supposed that diamino-compounds, as we have assumed for albumoses, serve in the formation of proteids.

(7) Nitrogen of monoamino-compounds. The increase of monoamino-compounds in the two kinds of plants at the 12-days stage does not show much difference but its amount is about twice that of the 6-days stage, as shown in Table III. Apparently monoamino-compounds are formed as by-products of proteid synthesis; but the conception of continuous translocation of this group from the cotyledon to the stem offers some objections to any such assumption. The following table shows the total nitrogen of monoamino-bodies in 1000 plants (cotyledon and stem).

	6-days etiolated plant.	12-days etiolated plant.	12-days green plant.
Nitrogen of monoamino-bodies in 1000 cotyledons	5.54 gm	2.56 gm	1.98 gm
Nitrogen of monoamino-bodies in 1000 stem	4.94 "	9.65 "	9.60 "
Total	10.48 "	12.21 "	11.58 "

In Table III, the fluctuation of amounts of insoluble and of coagulable proteids in the 12-days etiolated and green plants appears to have great influence on the values for albumoses, peptones and diamino-compounds but not on the value for monoamino-compounds. It suggests the idea that certain monoamino-bodies unprecipitated by phosphotungstic acid may not be so active in the synthesis of proteids.

(8) Nitrogen of ammonia. This was estimated with the 6-days plants in fresh condition but the 12-days plants were analyzed after drying at 50–60° C. Consequently the results on the latter, as E. Schulze shows, should be high. Assuming that the results obtained are higher than the actual weights, ammonia still plays an active part in the germination, although we are uncertain whether it is directly concerned in proteid synthesis or aids in the formation of amino-compounds.

SUMMARY.

I. Cotyledon.

(1) In the cotyledon, all proteids except peptones show a decrease at the 6-day and 12-day stages of growth which is most conspicuous in the case of coagulable proteids.

(2) Peptones, diamino-bodies, monoamino-bodies and ammonia show an increase at the 6 days stage and after that they decrease, especially in the cotyledon of green plants.

(3) The increase of these substances must be due to the decomposition of higher proteids.

(4) The decrease of all nitrogenous substances at the 12 days stage must be due to a translocation into the stem. Different amounts of decrease for the two 12-day stages, in darkness and in sunlight, satisfactorily explain the different degrees of growth of etiolated and of green stems.

II. Stem.

(1) In the stem of the 12 days etiolated plant, all of the nitrogen compounds show an increase, with the exception of diamino-bodies, in comparison with those of the 6 days etiolated plant; and the increase of insoluble proteids over the amounts of this form of proteids at the 6-day stage is most remarkable.

(2) Table III shows that the amount of insoluble and coagulable proteids in the stem of the 12-day green plant is noticeably higher than those for the stems of 12 days etiolated plants. It is evident, therefore, that the formation of insoluble and coagulable proteids is more active in sunlight than in darkness, causing the decrease of albumoses, peptones and diamino-compounds.

(3) The phenomenon of parallelism in the fluctuation of values for peptones and diamino-compounds in the cotyledon at every stage of germination and growth is striking, while of equal interest is the relation shown between the amounts of peptones and albumoses in the stem; peptones from the standpoint of molecular complexity, probably having a position between albumoses and diamino-bodies.

This work was done under the direction of Prof. E. B. Hart, to whom the writer wishes to express his sincere thanks.

ON THE CHEMICAL PROPERTIES OF AMANITA-TOXIN.

By HERMANN SCHLESINGER and WILLIAM W. FORD.

(*From the Pharmacological Laboratory of the Johns Hopkins University.*)

(Received for publication, July 1, 1907.)

It was pointed out by Kobert[1] in 1899 that *Amanita phalloides* contains, in addition to the hæmolytic "toxalbumin," Phallin, described by him several years previously as the active principle of this poisonous fungus, an alcohol-soluble substance killing small animals acutely after subcutaneous administration of minute doses. The second poison Kobert stated to be an alkaloid, without, however, specifying any of its alkaloidal reactions, but because of its failure to produce fatty degeneration of the parenchymatous organs, the most striking lesion seen in fatal cases in man, he could not accept it as the active principle.

At the same time he admitted that Phallin is not constantly present in the plant and can therefore be no longer looked upon as the essential poison. It was subsequently claimed by one of us (Ford[2]) as the result of inoculating animals with extracts of the fungus heated to 80° C. and subjected to artificial digestion, whereby the hæmolysins are completely destroyed, that a powerful toxin is present in this plant, in addition to the blood-laking substance, "Phallin."

This heat-resistant substance was called provisionally *Amanita-toxin.*

Recently Abel and Ford[3] have shown that Phallin is not a "toxalbumin" as supposed by Kobert, but a hæmolytic glucoside, which is so sensitive to heat, and to a hydrochloric-acid-pepsin mixture as to preclude its playing any rôle in ordinary human intoxications. If the aqueous extract of the fungi be treated with ethyl alcohol, this hæmolysin is precipitated while the heat resistant *Amanita-toxin* is found in the alcohol filtrate.

[1] *Sitzungsber. d. naturforsch. Gesellsch. zu Rostock*, p. 26, 1899.

[2] *Journ. of Exp. Med.*, viii, No. 3, May 26, 1906.

[3] This *Journal*, ii, No. 4, January, 1907.

Abel and Ford suggest that the *Amanita-toxin* is without doubt the active principle of *Amanita phalloides*, probably identical with the poison roughly studied by Kobert and assumed by him, on insufficient grounds, to be an alkaloid.

Finally it has been shown by Ford[1] that the heat resistant alcohol-soluble *Amanita-toxin* when completely freed from the hæmolytic glucoside, can produce in animals the lesions found in man, including the *hæmorrhage*, *necrosis*, and especially the *fatty degeneration*. It remains necessary, therefore, to take up more fully the question of the purification of this *Amanita-toxin*.

One hundred grams of fungi, previously dried over sulphuric acid, were finely ground and the powdered material thoroughly triturated with 300 cc. of 65 per cent ethyl alcohol. The residue was twice treated in the same way and finally mixed with 100 cc. of alcohol of the same strength and allowed to stand over night. The liquid of this fourth extraction was united with the first three fractions, making a total of 1000 cc. After careful neutralization with sodium carbonate the extract was evaporated under diminished pressure until all the alcohol had been distilled away and the volume of the remaining fluid had become reduced to 150–200 cc. After filtering from deposited fatty acids, etc., the solution was made very slightly alkaline with sodium carbonate and treated with a 15 per cent solution of silver nitrate. A voluminous precipitate was formed. This, being non-toxic, was discarded. The filtrate was freed from the slight excess of silver by means of sodium chloride and, now neutral, was treated with a solution of basic lead acetate prepared in the usual way. Another non-toxic precipitate was formed and was also discarded. The filtrate[2] from basic lead acetate was treated with an excess of a saturated sodium sulphate solution for the removal of lead and to this filtrate phosphotungstic acid (10 per cent phosphotungstic acid in 5 per cent sulphuric acid) was added in slight

[1] Paper to be published shortly.

[2] Sometimes a considerable amount of toxic material was included in the first lead precipitate. This should, therefore, be treated with a saturated sodium sulphate solution and filtered. The filtrate is again precipitated with basic lead acetate. The precipitate may now be discarded and the new filtrate treated as described above.

excess. The phosphotungstic precipitate was decomposed with barium hydrate and the filtrate from the barium compounds neutralized with sulphuric acid. The precipitate thus formed was filtered off and the resulting fluid found to contain the poisonous substance. On subcutaneous inoculation of both rabbits and guinea pigs this fluid was highly toxic, 1 cc., containing 0.0004 gram of organic and no inorganic material, killing the animals acutely in from 24–48 hours and producing the pathological changes characteristic of poisoning by *Amanita-toxin*.

It will be observed that our toxic solution as thus obtained is the product of a rather rigorous analytical separation. The final fluid can only contain substances precipitable by phosphotungstic acid, which at the same time do not form insoluble compounds with either silver or lead and which are soluble in 65 per cent alcohol. The material must, therefore, be freed from ordinary plant constituents. We nevertheless found it useful to repeat the precipitation by phosphotungstic acid several times in order to eliminate any error due to included matter. With our purified poisonous product we were able to establish the following points in regard to its chemical properties.

Amanita-toxin is very soluble in water, less so in 80 per cent alcohol and only very little soluble even in hot absolute alcohol; it is insoluble in the ordinary organic solvents. Its aqueous solution is optically inactive. It is a fairly stable compound for it can be boiled in absolute alcohol and in aqueous solution for some time without suffering serious loss in toxicity; it is only very slowly affected by acids at room temperature, retaining its toxicity for several days when thus treated. Boiling acids, however, rapidly destroy the poison. It does not reduce Fehling's solution either before or after prolonged boiling with 5 or 10 per cent hydrochloric acid. With the exception of phosphotungstic acid, this toxin reacts with none of the alkaloidal precipitants, nor does it respond to any of the alkaloidal color reagents.[1] It does not give the biuret test or Millon's reaction. We may, therefore, conclude that this poison is neither a glucoside, an alkaloid, nor a proteid in the generally accepted sense

[1] For a list of these, see Kippenberger, *Nachweis von Giftstoffen*.

of these terms. The following reactions give us a clue to its identity, and we are convinced that these reactions are due to the *Amanita-toxin* itself because of our rigorous method of purification and because the tests become more pronounced as the process of purification advances. Fusion with metallic potassium and subsequent treatment in the usual fashion shows the presence of *nitrogen* and *sulphur*. By boiling a concentrated solution of the purified toxin with hydrochloric acid and subsequently treating it with barium chloride the sulphur was shown to be present as conjugate sulphuric acid.[1] While making the fusion with potassium a strong odor of fatty amines was observed, and the gas evolved gave white fumes when a drop of hydrochloric acid on a glass rod was brought near. To determine whether the toxin is a substance from which amines may be split off by reagents ordinarily used for this purpose a small portion of the dried material[2] was mixed in a test-tube with powdered potassium hydrate. The amine odor was noticeable at once, but after heating, the persistent and unmistakable odor of *indol* completely masked that of the amines and a pine splinter moistened with concentrated hydrochloric acid gave the characteristic pyrrol red when held in the mouth of the test tube. The application of the tryptophan test of Hopkins and Cole gave negative results.

CONCLUSIONS.

From the reactions described above *Amanita-toxin* can not be a proteid, a glucoside, or an alkaloid. Any attempts to classify it definitely are made upon the assumption that the decomposition products we obtained are not due to very small amounts of impurity but come from the toxin itself. Because we were unable to obtain indol by boiling with concentrated solutions of potassium hydrate, we cannot be sure that we have an indol or pyrrol derivative. Nevertheless, since we are dealing with a conjugate sulphate which, on fusion with dry potassium hydrate, gives off pyrrol and indol we are led to conclude that *Amanita-*

[1] The solution gave no test for sulphates before boiling.

[2] Boiling with a *solution* of potassium hydrate gave no noticeable amine odor or alkaline fumes.

toxin, although not necessarily an indol derivative, is at least an aromatic phenol so combined with an amine group that it readily forms an indol or pyrrol ring.

We wish to take this occasion to thank Dr. Abel for placing material for this investigation at our disposal and for his many valuable suggestions during the progress of the work.

V. RESEARCHES ON PYRIMIDINS: ON SOME SALTS OF CYTOSIN, ISOCYTOSIN, 6-AMINOPYRIMIDIN AND 6-OXYPYRIMIDIN.

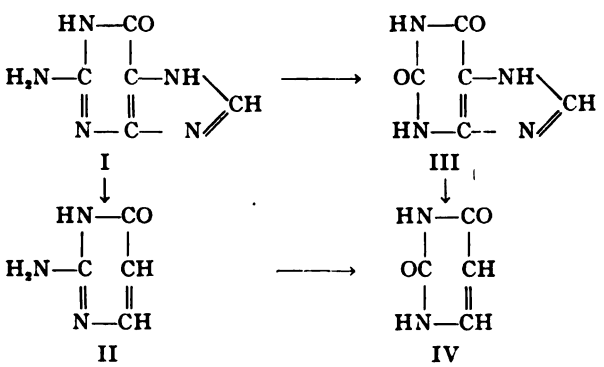
(Twenty-second Paper.)

By HENRY L. WHEELER.

(*From the Sheffield Laboratory of Yale University.*)

(Received for publication, June 10, 1907.)

It has been shown by Richard Burian[1] when guanin (I), mixed with carbohydrates, is heated with 30–40 per cent sulphuric acid that hydrolysis and reduction take place at the same time, the imidazole group is removed and isocytosin[2] (II) and uracil (IV) result. It may be added that the probable formation and decomposition of xanthin (III) would also give uracil. This decomposition of guanin may be represented as follows:

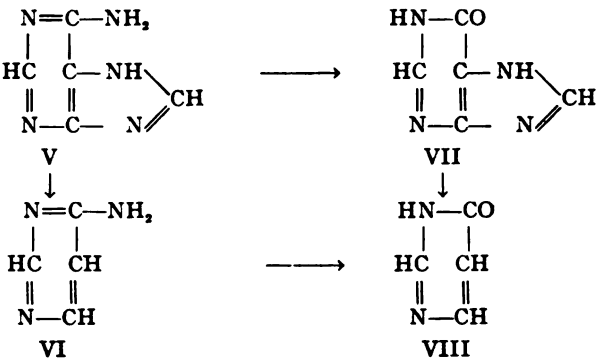


When adenin (V) was treated in a similar manner, Burian obtained 6-aminopyrimidin (VI), while 6-oxypyrimidin (VIII), which would be expected to result in this case both by the

[1] Asher-Spiro: *Ergeb. d. Physiol.*, v, p. 795, 1905.

[2] Wheeler and Johnson: *Amer. Chem. Journ.*, xxix, p. 492, 1903.

decomposition of hypoxanthin (VII) and by the hydrolysis of 6-aminopyrimidin, escaped detection.[1]



The three pyrimidin bases, isocytosin, 6-aminopyrimidin and 6-oxypyrimidin, are probably formed in the energetic hydrolysis of the nucleic acids by sulphuric acid and, since it has now been found that the general reagents which precipitate cytosin also precipitate these bases, an examination of the properties of the compounds and some of their salts was undertaken, along with the similar ones of cytosin. The statement of Burian that 6-aminopyrimidin and isocytosin according to their entire behavior must obstinately adhere to cytosin ("dem sie ihrem ganzen Verhalten nach hartnäckig anhaften müssten") has been found to be more especially true in the case of isocytosin.

Of the three bases 6-oxypyrimidin is new. Isocytosin was first prepared synthetically in this laboratory[2] and later it was obtained in a different manner by Gabriel and Colman.[3]

In the case of 6-aminopyrimidin, Burian states that the base was isolated in the form of the silver salt, by precipitating in neutral solution with silver nitrate. This was decomposed by means of hydrogen sulphide and the base precipitated by phosphotungstic acid. A solution of the free base was then obtained in the usual manner from which he prepared and analyzed the

[1] In an article published while this paper was in press (*Zeitschr. f. physiol. Chem.*, li, p. 444, 1907), Burian describes the isolation of 6-oxypyrimidin.

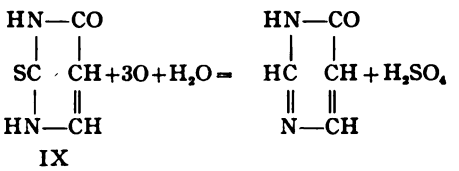
[2] *Loc. cit.*

[3] *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 3382, 1903.

picrate and chloroplatinate. He says nothing further in regard to the base or its salts.

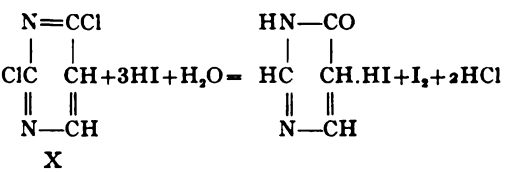
6-Aminopyrimidin, however, was first prepared by Ernst Büttner, who obtained it from barbituric acid by means of a series of operations.[1] This author described the free base and he prepared the hydrochloride, easily soluble rhombic tables; the chloroplatinate and picrate, needles, difficultly soluble. Our knowledge of these pyrimidins stood at this point when the following work was begun.

It has now been found that 6-oxypyrimidin is formed when 2-thiouracil (IX), which can be easily prepared[2] in any desired quantity, is treated with hydrogen dioxide.



IX

The writer finds, however, that 6-oxypyrimidin is more smoothly obtained by warming 2, 6-dichlorpyrimidin (X), prepared from uracil[3] or 2-thiouracil,[4] with hydriodic acid and red phosphorus. The hydrogen iodide salt results from which the pure base can be obtained in the usual manner.



X

The 6-aminopyrimidin used in this work was prepared by a shorter and more convenient method than the one employed by Büttner mentioned above. 2, 6-Dichlorpyrimidin[5] was heated

[1] *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2232, 1903.

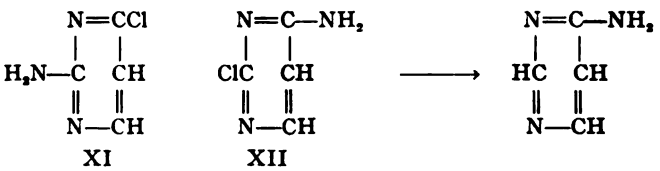
[2] Wheeler and Bristol: *Amer. Chem. Journ.*, xxxiii, p. 458, 1905.

[3] Gabriel: *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 1690, 1905.

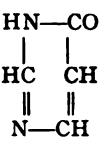
[4] Johnson and Menge: This *Journal*, ii, p. 114, 1906.

[5] Care should be taken in working with 2, 6-dichlorpyrimidin not to expose the material to the skin, since it has a very corrosive action. In one case deep and painful blisters were formed on the hands, resembling those produced by hydrofluoric acid.

with alcoholic ammonia. This gave a mixture of 2-amino-6-chlorpyrimidin (XI) and 2-chlor-6-aminopyrimidin (XII).[1] It was found, on boiling this mixture with water and zinc dust. that 2-amino-6-chlorpyrimidin was reduced to the very soluble 2-aminopyrimidin[2] which could easily be removed, while 2-chlor-6-aminopyrimidin remained unaltered. The pure 2-chlor-6-aminopyrimidin then on warming on the steam-bath with hydriodic acid was smoothly reduced and the hydrogen iodide salt of 6-aminopyrimidin was obtained. Although Büttner worked with small quantities of this base (0.25 gram) his results and the writer's agree in every respect.



EXPERIMENTAL PART.

6-Oxypyrimidin, 

Thirteen grams of 2,6-dichlorpyrimidin were slowly added to 50 cc. of concentrated hydriodic acid and 6 grams of red phosphorus on the steam-bath. As soon as all was added the mixture was boiled for a few minutes and then the hydrogen iodide was removed, as far as possible, by evaporation in a vacuum at 100°. The residue on taking up in hot water and filtering from red phosphorus formed a syrup, which deposited well crystallized needles, decomposing with effervescence when kept at 300° (6-oxypyrimidin hydriodide). The whole was dissolved in water and an excess of silver sulphate was added and filtered, the filtrate was precipitated with hydrogen sulphide, the phosphoric and sulphuric acids removed by means of barium

[1] Gabriel: *Loc. cit.*
[2] Büttner: *Loc. cit.*

hydroxide, and then, on removing the excess of barium with carbon dioxide and evaporating to dryness, a very soluble crystalline cake was obtained. This weighed 5.6 grams. On crystallizing from ethyl acetate beautiful, long, thin, prismatic needles separated melting to a clear oil at 164°–165°. The per cent of nitrogen in this material agreed with the calculated for 6-oxypyrimidin (Analysis I and II).

The same compound was also obtained as follows: Twenty grams of 2-thiouracil were suspended in a liter of hot water and about 530 cc. of commercial hydrogen dioxide solution were added in portions. The thiouracil dissolved and after boiling a few minutes, sulphur dioxide was added and the solution was evaporated to a convenient volume. The sulphuric acid, which had been formed in the reaction, was removed by means of an excess of barium hydroxide and the excess of the latter was precipitated with carbon dioxide. The solution was then evaporated to dryness and the residue was extracted with boiling alcohol. This dissolved the 6-oxypyrimidin, leaving a mixture weighing about 7.7 grams of uracil and a barium salt of an organic acid that was not further examined. The barium salt was insoluble in alcohol but readily soluble in water, it had the peculiar property of swelling up to many times its original volume when heated on platinum foil.

The alcoholic solution was evaporated to dryness and the residue extracted with ethyl acetate. This gave about 6 grams of crude 6-oxypyrimidin. When crystallized from a large amount of benzene it formed colorless needles melting less sharply than the above preparation (Analysis III). All the nitrogen determinations in this paper were made by Kjeldahl's method.

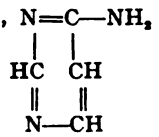
	Calculated for $C_4H_4ON_2$:	Found: I.	II.	III.
N	29.16	29.02	28.78	28.85

6-Oxypyrimidin is insoluble in petroleum ether, very difficultly soluble in ether, somewhat more soluble in hot benzene and still more soluble in ethyl acetate. This solvent is the best to use to extract and crystallize the material. It is extremely soluble in water and alcohol, and, in this respect, like 6-aminopyrimidin could not possibly be mistaken for the far more insoluble cytosin and isocytosin.

This monoöxypyrimidin unlike the dioxypyrimidin (uracil) gives no color with bromine and barium hydroxide.

It is precipitated by phosphotungstic acid, and, in neutral solution, by silver nitrate or mercuric chloride. A strong solution is precipitated by picric acid and also by hydrochloroplatinic acid. The platinum chloride double salt separates slowly and forms prisms. It did not have a definite melting or decomposing point.

6-Aminopyrimidin,



2-Chlor-6-aminopyrimidin reduces smoothly to 6-aminopyrimidin when warmed on the water-bath with concentrated hydriodic acid and red phosphorous.[1] The residue, after the removal of hydrogen iodide by evaporation was treated with silver sulphate, barium hydroxide, etc., as in the case of 6-oxypyrimidin, for the preparation of the free base. It was found that 2.7 grams of 2-chlor-6-aminopyrimidin, 27 cc. of concentrated hydriodic acid and an excess of red phosphorus gave 1.6 gram of crude base while the calculated is 1.9 gram. When crystallized from ethyl acetate, snow white clusters of thin, leaf-like crystals separated. The appearance of these clusters was similar to those of 6-oxypyrimidin. The material melted at 151°–152°. Büttner[2] gives the same melting point for this base. It is extremely soluble in water and alcohol and it is precipitated in more dilute solutions by the same reagents which precipitate 6-oxypyrimidin.

The Acetyl Compounds.

A peculiar acetyl derivative of 6-oxypyrimidin was formed when the base was dissolved in acetic anhydride and evaporated to dryness on the steam-bath. When the residue was crystallized from alcohol, in which it is quite soluble, it formed colorless needles or spikes. It is sharply distinguished from the other

[1] Wheeler and Johnson: This *Journal*, iii, p. 186, 1907.
[2] *Loc. cit.*

acetyl compounds by having two melting points. When heated rapidly it melted to a clear oil at 180°, or a little below, then if the temperature was kept at this point it solidified and on further heating it remelted with effervescence at 215°–220°. (Analysis I and II.)

Another sample of the acetyl compound was prepared and crystallized from water. It then separated in the form of prismatic scales which had the same behavior on heating as the above. It was dried at 50°–55°.

(Analysis III.) The analytical results are low for a simple acetyl derivative, but they agree with the calculated for the expected acetyl compound with a molecule of water of crystallization, or, if water of constitution, equally well for acetylformamidine acrylic acid, $CH_3CONH-CH=N-CH=CHCOOH$, or simply an acetic acid salt of 6-oxypyrimidin. The latter, however, is excluded since the free base dissolves in glacial acetic acid and on evaporation is recovered unaltered.

In order to determine whether the compound has water of crystallization or water of constitution, a portion of the material, which had been crystallized from water, was heated at 109°–115° for three hours. It then lost 4 per cent in weight This was due, not to the fact that water was given off but that the substance volatilized, since a nitrogen determination, after heating, gave the same result as in the case of the previous determinations (Analysis IV). This result makes it appear improbable that the substance has water of crystallization. The view that the compound is acetylformamidine acrylic acid, therefore, remains at present as most probable. This, however, must be left for future work to decide.

	Calculated for $C_6H_8O_2N_2$:	Found: I.	II.	III.	IV.
N	17.94	17.75	17.64	17.88	17.86

Acetyl-6-aminopyrimidin.—A quarter of a gram of the pure base was dissolved in acetic anhydride and heated to boiling, then evaporated to dryness on the steam-bath When crystallized from about 5 cc. of water it formed an asbestos-like mass of fine needles. About 0.2 gram separated. It melted at 202°, to a clear oil, without effervescence. Analysis:

	Calculated for $C_6H_7ON_3$:	Found:
N	30.43	30.35

The Picrates.

The picrate of 6-oxypyrimidin is far more soluble than the picrates of cytosin and isocytosin. It also differs decidedly in appearance from these salts. 6-Aminopyrimidin picrate, on the other hand, closely resembles cytosin picrate both in regard to solubility and crystalline form. The presence of this picrate is possibly the cause of the picrate of cytosin from natural sources invariably melting lower than that of synthetic cytosin.[1]

6-*Oxypyrimidin Picrate.*—A saturated aqueous solution of picric acid was mixed with a moderately strong solution of 6-oxypyrimidin; as no precipitate was formed the solution was concentrated to almost the volume of picric acid solution employed. On standing a long, flat, fern-like growth of crystals separated. It melted to a clear oil at 190°. Analysis:

	Calculated for $C_{10}H_7O_8N_5$:	Found:
N	21.53	21.55

6-*Aminopyrimidin Picrate.*—Forty cc. of picric acid solution were added to 0.2 gram of 6-aminopyrimidin in a little water. A bulky precipitate was formed at once which dissolved on adding 40 cc. of water and then boiling. On cooling, long bright, yellow, hair-like needles separated. On heating these showed evidence of change a little below 200° and then suddenly melted at 226° to a clear oil. This then turned brown and vigorously effervesced at 270°–280°. Analysis:

	Calculated for $C_{10}H_8O_7N_6$:	Found: I.	Found: II.
N	25.92	25.76	25.92

The Hydrochlorides.

The hydrochlorides of 6-oxypyrimidin, 6-aminopyrimidin and of isocytosin are even more soluble than the easily soluble cytosin hydrochloride. The latter and 6-oxypyrimidin hydrochloride separate with water of crystallization. The hydrochlorides are difficultly soluble in alcohol.

6-*Oxypyrimidin Hydrochloride*, $C_4H_4ON_2.HCl.H_2O$.—Pure 6-oxypyrmidin was dissolved in dilute hydrochloric acid and evaporated to dryness on the steam-bath. The residue formed a syrup

[1] *Amer. Chem. Journ.*, xxix, pp. 494, 500, 505, 1903.

which solidified on cooling. It was dissolved in water and allowed to crystallize by standing over sulphuric acid. Thick transparent prisms or oblong blocks separated some 10–15 millimeters in length. These melted partially below 100°, finally melting to an oil at about 205°–210°. Analysis:

	Calculated for $C_4H_7O_2N_3Cl$:	Found: I.	Found: II.
N	18.60	18.54	18.64

A water determination was made by heating the material at 114° for one hour. This gave 13.1 per cent while the calculated is 11.96. When reheated at 120° it was found that the material slowly volatilized, which explains the high result.

6-*Aminopyrimidin Hydrochloride*, $C_4H_5N_3.HCl$.—The base was evaporated to dryness with hydrochloric acid. The material was then taken up in a little water and left to crystallize in a desiccator. Transparent prisms or tables separated from the syrupy solution. When dried over calcium chloride they melted to an oil at 257° and then effervesced. A nitrogen determination showed that the salt was anhydrous.

	Calculated for $C_4H_5N_3.HCl$:	Found:
N	31.93	31.49

Isocytosin Hydrochloride, $C_4H_5ON_3.HCl$.—This salt has a decided tendency to crawl up the sides of a dish when left to crystallize. The crystals which separate in this manner are prisms, when precipitated from an aqueous solution by the addition of alcohol it forms little square tables or blocks. When heated it begins to change in appearance at about 250° and then effervesces about 270°. Analysis:

	Calculated for $C_4H_5ON_3.HCl$:	Found:
N	28.47	28.27

Cytosin Hydrochloride, $C_4H_5ON_3.HCl.H_2O$.—If cytosin is dissolved in strong hydrochloric acid and left to crystallize in a desiccator cytosin dihydrochloride is obtained, $C_4H_5CN_3 2HCl$.[1] If the acid solution is evaporated to dryness and the residue is taken up in water and left to crystallize spontaneously, large, transparent plates separate of the hydrous, 1 : 1 salt. This salt

[1] Wheeler and Johnson: *Amer. Chem. Journ.*, xxxi, p. 598, 1904.

loses its water rapidly at 50° and in a few hours on exposure to the air. It differs from the hydrous 6-oxypyrimidin hydrochloride since on standing over night the crystals become entirely opaque. It is more soluble than the dihydrochloride. The latter and also the hydrous cytosin hydrochloride both melt at 275°–279°. Analysis:

	Calculated for $C_4H_5ON_3.HCl.H_2O$:	Found:
N	25.37	25.52

Some of the material which had stood for about 3–4 hours was dried to a constant weight at a little above 100°. It lost 9.5 per cent of water while the calculated for one molecule of water is 10.8 per cent.

The Sulphates.

The sulphates of 6-oxypyrimidin and 6-aminopyrimidin are very soluble in water. The neutral sulphate of isocytosin is less soluble and it resembles the neutral sulphate of cytosin except that it does not crystallize with water. The three sulphates which cytosin forms have been prepared and some new facts are given for their identification. The sulphates were prepared from the hydrochlorides by treating the latter with an excess of silver sulphate, removing the excess of silver by means of hydrogen sulphide and then evaporating. Owing to their solubility, the sulphates of 6-oxypyrimidin and 6-aminopyrimidin were crystallized by means of alcohol. The sulphates of isocytosin and cytosin were allowed to crystallize at ordinary temperatures in order to determine their degree of hydration.

6-*Oxypyrimidin Sulphate*, $(C_4H_4ON_2)_2H_2SO_4$.—This salt was purified by precipitating the strong aqueous solution with alcohol. After the fourth precipitation it came down in the form of microscopic prisms and melted with effervescence about 218°. Analysis:

	Calculated for $C_8H_8O_2N_4.H_2SO_4$:	Found: I.	Found: II.
N	19.31	19.29	19.32

This neutral sulphate also separates from alcoholic solutions containing a considerable excess of sulphuric acid.

6-*Aminopyrimidin Sulphate*, $C_4H_5N_3.H_2SO_4$, was described in a previous paper.[1]

Isocytosin Sulphate, $(C_4H_5ON_3)_2H_2SO_4$.—This salt separated from the aqueous solution containing a slight excess of sulphuric acid, on standing, in the form of balls composed of radiating clusters of small prisms. It melted at 276° with effervescence. Analysis:

	Calculated for $C_8H_{10}O_2N_6.H_2SO_4$:	Found: I.	Found: II.
N.....	26.25	26.30	25.98

Basic Cytosin Sulphate, $(C_4H_5ON_3)_4H_2SO_4.2H_2O$.—This hydrous salt has frequently been obtained by Levene.[2] It was obtained in the anhydrous condition by Kossel and Steudel.[3] It is the least soluble of the sulphates mentioned in this paper. It has the highest decomposing point, the same as that of cytosin itself, namely, 323°. It was obtained, in the present work, when cytosin monohydrochloride was treated with a slight excess of silver sulphate, and the silver then removed by means of hydrogen sulphide. On concentrating the solution the salt separated in the form of sharply defined, long, needle-like prisms.

Neutral Cytosin Sulphate, $(C_4H_5ON_3)_2H_2SO_4.2H_2O$.—The anhydrous form of this salt was first obtained by Levene,[4] having dried the material in a toluol bath. The hydrous form, here described, separated on allowing the mother liquor from the above basic salt to evaporate in the air. Stouter, more compact masses of prisms formed which, on drying in the air, melted with effervescence at 287°. Levene found 290°. The analyses now show that this salt crystallizes with two molecules of water.

	Calculated for $C_8H_{10}O_2N_3.H_2SO_4.2H_2O$:	Found: I.	Found: II.	Found: III.
N.....	23.59	23.66	23.45	
H_2O	10.11			10.13

The water determination was made by heating the salt at 118°–120° for an hour.

Acid Cytosin Sulphate, $C_4H_5ON_3.H_2SO_4$.—This salt has been obtained by Kossel and Steudel; they simply mention that it is

[1] Wheeler and Johnson: This *Journal*, iii, p. 189, 1907.

[2] *Zeitschr. f. physiol. Chem.*, xxxix, pp. 7, 135, 481, 1903.

[3] *Ibid.*, xxxviii, p. 52, 1903.

[4] *Ibid.*, xxxviii, p. 81, 1903.

easily soluble.[1] It is easily obtained by dissolving the neutral sulphate in 20 per cent sulphuric acid and allowing the material to crystallize in a desiccator. The stout, transparent crystals thus obtained appear to be rhombohedrous. These have the characteristic property of becoming opaque when an attempt is made to wash them with water. This is the most soluble sulphate of cytosin. After pressing on paper and drying over calcium chloride the material melted at 197° to a colorless oil. Analysis:

	Calculated for $C_4H_5ON_3.H_2SO_4$:	Found: I.	Found: II.
N.....	20.09	19.86	19.89

Acid Cytosin Phosphate, $C_4H_5ON_3.H_3PO_4$.—This salt was obtained when cytosin monohydrochloride (7 grams) was boiled with phosphorous oxychloride (50 cc.) for two hours. The phosphorous oxychloride was then evaporated and the residue treated with ice. The material dissolved and, evaporating, a syrup was obtained from which dilute alcohol precipitated well crystallized, long, flat prisms. When crystallized from water, in which the salt is very soluble, it melted at 236° with effervescence. Analysis:

	Calculated for $C_4H_5ON_3.H_3PO_4$:	Found: I.	Found: II.
N.....	20.09	20.09	19.95

The melting or effervescing points of the substances compared in this work are given in the following table. The effervescing points even of the pure compounds may vary to a certain amount, perhaps several degrees, according to the rate of heating, amount of substance used, etc. No especial accuracy is claimed. It is believed, however, that the melting points given will be found to be sufficiently constant to serve as an aid for the identification of the substances. There is in general a wide difference in the melting points in each series. This should be especially serviceable in the case of the sulphates of cytosin.

The picrolonates mentioned in the table are the most insoluble salts prepared. Since some new facts have been observed in the case of picrolonates these salts will be described in a later paper.

[1] *Zeitschr. f. physiol. Chem.*, xxxviii, p. 52, 1903.

	Base	Acetyl Derivative	HCl Salts	H_2SO_4 Salts	Picrate	Picrolonate
HN—CO | | HC CH ‖ ‖ N—CH 6-oxypyrimidin	164°*	180°* 215°	$C_4H_4ON_2.HCl.H_2O$* 205°-210°	$(C_4H_4ON_2)_2H_2SO_4$* 218°	190°*	193°
N=C—NH_2 | | HC CH ‖ ‖ N—CH 6-aminopyrimidin	151°*	202°*	$C_4H_5N_3.HCl$* 257°	$C_4H_5N_3.H_2SO_4$** 143°	226°*	261°
HN—CO | | H_2N—C CH ‖ ‖ —CH isocytosin	276°†‡‡	247°†‡‡	$C_4H_5ON_3.HCl$* 270°	$(C_4H_5ON_3)_2H_2SO_4$* 276°	270°-280°†	273°-275°
N=C—NH_2 | | OC CH | ‖ HN—CH cytosin	323°†	Above 300°†	$C_4H_5ON_3.2HCl$‡ 275°-279° $C_4H_5ON_3.HCl.H_2O$* 275°-279°	$C_4H_5ON_3.H_2SO_4$* 197° $(C_4H_5ON_3)_2H_2SO_4.2H_2O$* 287°-290° †† $(C_4H_5ON_3)_4H_2SO_4.2H_2O$* 323°	Above 300°†	270°

* This paper. † Wheeler and Johnson *Amer Chem. Journ.* xxix p. 492, 1903. ‡ *Ibid.*, xxxi, p. 598, 1904.
** This *Journal*, iii, p. 183, 1907 †† Levene: *Zeitschr. f. physiol. Chem.*, xxxviii, p. 81, 1903.
‡‡ Gabriel and Colman: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 3382, 1903.

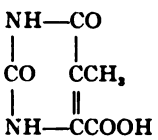
VI.—RESEARCHES ON PYRIMIDINS: SYNTHESIS OF THYMIN-4-CARBOXYLIC ACID.

By TREAT B. JOHNSON.

(*From the Sheffield Laboratory of Yale University.*)

(Received for publication, June 27, 1907.)

I shall describe, in this paper, the preparation and properties of thymin-4-carboxylic acid,



The study of the carboxylic acids of uracil, cytosin and thymin is of interest on account of the possibility that these pyrimidins may be linked in nucleic acids by acid amide groupings, —CO.NH. It has already been shown[1] that uracil may exist in nucleic acids as a 5-carboxyl compound since uracil-5-carboxylic acid can be quantitatively converted into uracil if heated with 20 per cent sulphuric acid at 160–170°. I now find that thymin-4-carboxylic acid can be heated with 20 per cent sulphuric acid, under the same conditions, without alteration. *Thymin therefore cannot exist in the nucleic acids as a 4-carboxyl compound.*

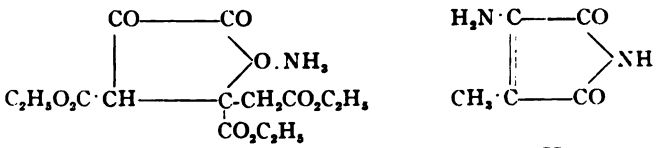
Wislicenus observed that diethyl oxalacetate and its homologues show a wide difference in their behavior towards ammonia and amines. Diethyl oxalacetate, for example, combines with ammonia to form an unstable addition product, which is changed to an ammonium salt of the lactone ester of oxalcitric acid (I)[2] when warmed with alcohol.

Diethyl methyloxalacetate, on the other hand, does not form

[1] *Amer. Chem. Journ.*, xxxvii, p. 392.

[2] Wislicenus and Beckh: *Ber. d. deutsch. chem. Gesellsch.*, xxviii, p. 789; *Ann. d. Chem.* (Liebig), ccxcv, p. 339.

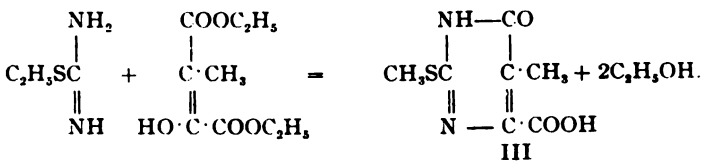
an addition product with ammonia but reacts at 110°, giving aminomethylmaleïnimide (II).[1]



I II

Another striking example of this difference between diethyl oxalacetate and diethyl methyloxalacetate was found when we investigated the behavior of pseudothioureas towards these esters. Pseudoethylthiourea combines with diethyl oxalacetate, giving a stable addition product (Professor Wheeler).

On the other hand, diethyl methyloxalacetate reacts with pseudomethylthiourea, in presence of potassium hydroxide, giving a good yield of the potassium salt of 2-methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin (III). The condensation can be represented as follows:



III

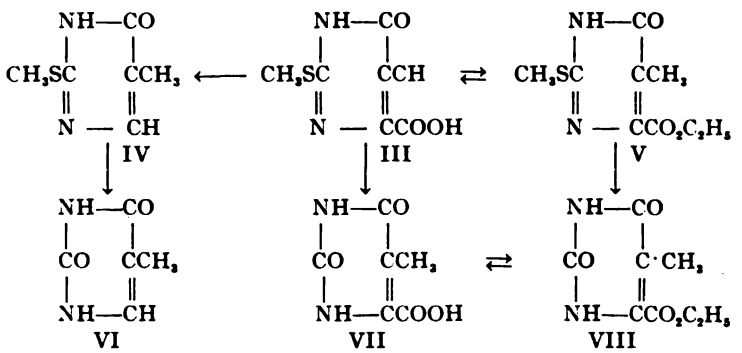
2-Methylmercapto-4-carbethoxy-5-methyl-6-oxypyrimidin (V) was obtained, in one experiment, as a secondary product of the condensation.

2-Methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin (III) can be converted into thymin-4-carboxylic acid (VII), by digesting with concentrated hydrochloric acid. When this acid was boiled, in ethyl alcohol solution, with a small quantity of sulphuric acid it was converted into thymin-4-ethylcarboxylate (VIII). This same ester was also obtained when 2-methylmercapto-4-carbethoxy-5-methyl-6-oxypyrimidin (V), was boiled in alcoholic solution with sulphuric acid. When thymin-4-carboxylic acid (VII), is melted it undergoes complete decom-

[1] Wislicenus and Kiesewetter: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 194.

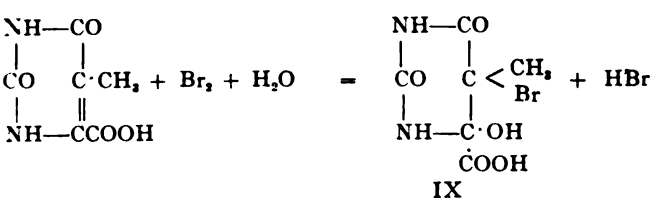
position. On the other hand, 2-methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin (III), melts with evolution of carbon-dioxide and is converted quantitatively into 2-methylmercapto-5-methyl-6-oxypyrimidin (IV). This mercaptopyrimidin can then be changed to thymin (VI) by boiling with hydrochloric acid.[1]

The formation of thymin in this manner from 2-methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin (III), shows that the condensation takes place as represented in the preceding equation and that the product is not a hydantoin derivative. The above compounds and their various transformations are represented as follows:



Thymin-4-carboxylic acid is characterized by its insolubility in cold water and by its property of crystallizing from aqueous solution with and without water of crystallization. It gives very insoluble barium and lead salts, and is not precipitated by phosphotungstic acid.

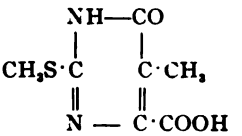
Thymin-4-carboxylic acid reacts in the normal manner with bromine water giving oxybromhydrothymin-4-carboxylic acid (IX).



[1] *Amer. Chem. Journ.*, xxix, p. 487.

EXPERIMENTAL.

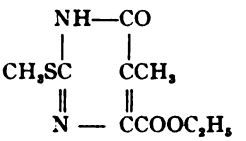
2-Methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin,



Thirty grams of pseudomethylthiourea hydriodide and 50 grams of the sodium salt of diethyl oxalpropionate were dissolved in about 500 cc. of water and two molecular proportions of potassium hydroxide (16 grams) added to the solution. The mixture was allowed to stand on the steam-oven for 8–10 hours and then concentrated to a volume of 150 cc. After thorough cooling, and acidifying with hydrochloric acid, the mercapto-pyrimidin deposited in prismatic crystals. It was practically insoluble in cold water and difficultly soluble in boiling water and alcohol. It separated from water or alcohol in rectangular prisms that melted at 243–244° with effervescence to a clear oil. It deposited from glacial acetic acid in stout prismatic crystals. The yield of acid corresponded to about 80 per cent of the theoretical. Analysis (Kjeldahl):

	Calculated for $C_7H_8O_3N_2S$:	Found:
N.....	14.00 per cent.	13.88 per cent.

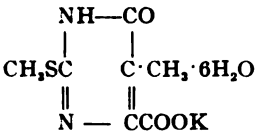
2-Methylmercapto-4-carbethoxy-5-methyl-6-oxypyrimidin,



This ester was obtained, associated with 2-methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin, when I used one instead of two molecular proportions of potassium hydroxide in the above condensation. It was difficultly soluble in cold water and alcohol, but deposited from hot alcohol or water in slender needles that melted at 201–202° to a clear oil without effervescence. Analysis (Kjeldahl):

	Calculated for $C_9H_{12}O_3N_2S$:	Found:
N.....	12.30 per cent.	12.37 per cent.

Potassium Salt of 2-Methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin,



A good yield of this salt was obtained under the following conditions: Pseudomethylthiourea and diethyl oxalpropionate were condensed as in the above experiment, in presence of two molecular proportions of potassium hydroxide. After standing for about twelve hours at 40–45° the solution was then acidified with acetic acid and concentrated to a volume of about 150 cc. On cooling the potassium salt deposited in distorted needles that decomposed with effervescence when heated above 230°. When this acetic acid filtrate was treated with hydrochloric acid the mercapto-acid separated melting at 243°. The potassium salt was very soluble in hot water and deposited, on cooling, in needles. They contained water of crystallization which was determined by heating at 100–110° for two hours.

1.2424 gram substance lost 0.4032 gram water.

	Calculated for $C_7H_7O_3N_2SK.6\,H_2O$:	Found:
H_2O.....	31.21 per cent.	32.4 per cent.

Nitrogen determination in the anhydrous salt (Kjeldahl):

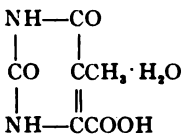
	Calculated for $C_7H_7O_3N_2SK$:	Found:
N.....	11.76 per cent.	11.91 per cent.

Behavior of 2-Methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin on Heating.—About two grams of the mercapto-acid were heated in a sulphuric acid bath at 245° until all effervescence ceased. I obtained a clear oil that crystallized, on cooling, in large, prismatic crystals. When these prisms were heated they melted sharply at 230–231° without effervescence to a clear oil. The compound deposited from water in flat prisms that melted at 233° and was identified as 2-methylmercapto-5-methyl-6-oxy-

pyrimidin.[1] When mixed with this pyrimidin the melting point was not lowered. Analysis (Kjeldahl):

	Calculated for $C_6H_8ON_2S$:	Found:
N.....	17.94 per cent.	17.80 per cent.

Thymin-4-carboxylic Acid,



A quantitative yield of this acid was obtained when 2-methylmercapto-4-carboxyl-5-methyl-6-oxypyrimidin was digested with concentrated hydrochloric acid. The oxygen acid separated from the acid solution as a granular powder that was difficultly soluble in boiling water and practically insoluble in alcohol. A most characteristic behavior of this acid is its property of crystallizing from hot water in anhydrous condition and with one molecule of water of crystallization. When a hot, saturated aqueous solution of the acid was allowed to cool slowly the anhydrous acid first deposited in balls of microscopic prisms resembling very much the crystalline form of uracil. They decomposed at 328–330° (Anschutz thermometer) and did not lose weight when heated at 120°. Analysis (Kjeldahl):

	Calculated for $C_6H_6O_4N_2$:	Found:
N.....	16.47 per cent.	16.37 per cent.

After filtering from the anhydrous acid and allowing the filtrate to stand, transparent, rectangular prisms of the hydrous acid deposited. They decomposed at the same temperature as the anhydrous acid (328–330°). The acid was not precipitated by phosphotungstic acid.

Water determination: 0.6742 gram substance lost 0.0687 gram water after heating one hour at 110–120°.

	Calculated for $C_6H_6O_4N_2.H_2O$:	Found:
H_2O.....	9.60 per cent.	10.1 per cent.

[1] *Amer. Chem. Journ.*, xix, p. 478.

Analysis of the anhydrous acid: 0.2592 gram substance gave 0.4002 gram CO_2 and 0.0854 gram H_2O. Nitrogen determination (Kjeldahl):

	Calculated for $C_6H_6O_4N_2$:	Found:
C.....	42.35 per cent.	42.11 per cent.
H.....	3.52 " "	3.66 " "
N.....	16.47 " "	16.23 " "

Action of 20 per cent Sulphuric Acid.—One-half a gram of the thymin acid was heated with 5 cc. of 20 per cent sulphuric acid for two hours at 160–170°. When the tube was opened there was no pressure and the unaltered acid was suspended in the sulphuric acid. I recovered 0.45 gram of acid melting at 326–329°. Analysis (Kjeldahl):

	Calculated for $C_6H_6O_4N_2$:	Found:
N.....	16.47 per cent.	16.15 per cent.

Potassium Salt, $C_6H_5O_4N_2K.2H_2O$.—This salt was prepared by dissolving molecular proportions of potassium hydroxide and thymin-4-carboxylic acid in water. When the solution was concentrated and allowed to stand for a few hours the salt deposited in radiating prisms. It was dried for analysis in a desiccator over sulphuric acid (Kjeldahl):

	Calculated for $C_6H_5O_4N_2K$:	Calculated for $C_6H_5O_4N_2K.2H_2O$:	Found:
N.....	13.45 per cent.	11.06 per cent.	10.97 per cent.

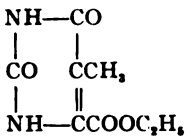
Lead Salt $(C_6H_5O_4N_2)_2$ Pb.—This salt deposited in well developed prisms when a solution of lead acetate was added to a hot, saturated, aqueous solution of thymin-4-carboxylic acid. It was practically insoluble in cold water. Analysis (Kjeldahl):

	Calculated for $(C_6H_5O_4N_2)_2Pb$:	Found:
N.....	10.27 per cent.	10.00 per cent.

Barium Salt $(C_6H_5O_4N_2)_2Ba$.—Thymin-4-carboxylic acid gives no precipitate when treated with a solution of barium chloride. The barium salt was prepared by dissolving the acid in potassium hydroxide and then adding the calculated amount of barium chloride. It separated from water in corpuscular crystals. Analysis (Kjeldahl):

	Calculated for $(C_6H_5O_4N_2)_2Ba$:	Found:
N.....	11.77 per cent.	11.30 per cent.

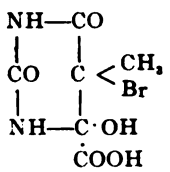
Thymin-4-ethylcarboxylate,



was prepared by esterifying the acid with ethyl alcohol and sulphuric acid. It was also obtained when 2-methylmercapto-4-carbethoxy-5-methyl-6-oxypyrimidin was boiled in alcoholic solution with a small amount of hydrochloric acid. The ester deposited from hot water in distorted prisms that melted at 255° to a clear oil without effervescence. Analysis (Kjeldahl):

	Calculated for $C_8H_{10}O_4N_2$:	Found:
N.....	14.14 per cent.	14.07 per cent.

Oxybromhydrothymin-4-carboxylic Acid,



Three and five-tenths grams of finely pulverized thymin-4-carboxylic acid were suspended in about 40 cc. of bromine water and bromine slowly added until the acid had completely dissolved. There was no evolution of carbon dioxide and when the solution was allowed to evaporate spontaneously in the atmosphere well-developed, prismatic crystals of the hydro-derivative separated. It was purified for analysis by recrystallization from bromine water. It crystallized in small, prismatic crystals that charred when heated above 270° and then decomposed with violent effervescence from 295–300° according to the rate of heating. When thymin-4-carboxylic acid was heated with bromine water at 146–152° it was completely decomposed with formation of bromoform. Analysis (Kjeldahl):

	Calculated for $C_6H_7O_3N_2Br$:	Calculated for $C_5H_5O_3N_2Br$:	Found: I.	II.
N.....	10.48 per cent.	12.66 per cent.	10.45	10.45 per cent.

THE BALANCE OF ACID-FORMING AND BASE-FORMING ELEMENTS IN FOODS.

(Preliminary Paper.)

By H. C. SHERMAN and J. EDWIN SINCLAIR.

(*Contribution from the Havemeyer Laboratories, Columbia University, No. 138*)

(Received for publication, June 19, 1907.)

Although mention is frequently made of the fact that some foods contain an excess of acid-forming and others of base-forming elements, no systematic quantitative study of the subject appears to have been published. It is true that the determination of the alkalinity of the ash is a method much used by food chemists in the examination of fruit products; but the alkalinity of the ash may easily be greater than would correspond to the excess of base-forming elements in the food itself, for even those food materials which yield an alkaline ash are apt to lose sulphur and chlorine in burning.

As early as 1885, Bunge[1] in reporting an analysis of beef flesh, made a partial computation which showed that the oxidation of the sulphur in the flesh would alone yield sufficient acid to combine with all of the bases present, and various writers have suggested that the injurious effects of "ash-free" food might be partly due to the lack of a sufficient supply of bases to neutralize the sulphuric acid produced in the protein metabolism, but so far as we are aware there has been no attempt at a systematic quantitative balancing of the acid-forming against the base-forming elements in foods, although similar work has been done with urine by several investigators. We have undertaken a study of this subject which, on account of the large amount of analytical work involved, will probably require a considerable time for completion. The present widespread interest in all subjects connected with acidosis suggests, however, a brief preliminary publication of some of the results already obtained.

[1] *Zeitschr. f. physiol. Chem.*, ix, p. 60.

While postponing detailed descriptions either of the materials examined or of the analytical methods employed, it may be stated that calcium, magnesium, sodium, and potassium were determined by the usual methods, and that chlorine, phosphorus and sulphur were determined in separate portions of the dried food material which were oxidized for the determination of chlorine by burning at a low temperature in the presence of a large excess of sodium carbonate; of phosphorus, by means of nitric and sulphuric acids and ammonium nitrate; of sulphur, by Liebig's method, checked by several determinations in which the oxidation was accomplished by burning in compressed oxygen.[1]

Of the different methods available for computing and expressing the results, the one which appears most satisfactory is to calculate the amount of each element found to the corresponding number of cubic centimeters of a normal solution of acid or base. By then adding together the results obtained for all of the base-forming and for all of the acid-forming elements, respectively, it is easy to compare the totals and the result obtained shows the excess of acid-forming or of base-forming elements in terms of a familiar standard and in figures of convenient magnitude.

For the purpose of this calculation, it is assumed that phosphoric acid is "neutralized" when two hydrogen atoms are replaced by bases. By this method of calculation the following results were obtained:

EXCESS OF ACID-FORMING OR OF BASE-FORMING ELEMENTS PER 100 GRAMS.

Food material in the fresh or dried state as ordinarily purchased.	Excess of acid-forming or of base-forming elements in terms of a normal solution of Acid: cc.	Base: cc.
Oatmeal (air-dry)	12.93	
Beef (fresh), free from visible fat	12.0	
Wheat, entire grain (air-dry)	9.66	
Milk, a composite sample		2.37
Peas (dried)		7.07
Prunes (air-dried)		24.4

The above figures may of course be as readily expressed on the basis of pounds or ounces and would possibly be most conven-

[1] Berthelot: *Compt. rend. de l'Acad. des sci.*, cxiv, p. 318; cxxix, p. 1002; Sherman: *Journ. of the Amer. Chem Soc.*, xxiv, p. 1100, and *Methods of Organic Analysis*, p. 19.

ient in that form for dietary computations based on weights of food material purchased or prescribed. It is probable, however, that a better impression of the relative acid-forming or base-forming tendencies of foods will be given if the final figures are expressed in terms of 100 calorie portions rather than 100 gram portions. The above figures then yield the following:

EXCESS OF ACID-FORMING OR OF BASE-FORMING ELEMENTS PER 100 CALORIES.

	Acid cc. Normal Solution.	Base cc. Normal Solution.
Beef, free from visible fat	10.1	
Oatmeal	3.15	
Wheat, entire grain	2.62	
Peas		1.94
Milk		3.31
Prunes		7.92

It is noticeable that peas, in spite of their high protein content, contain an excess of base-forming or acid-forming elements. Doubtless in most other vegetables this "potential alkalinity" will be found to be much greater. When a larger number of food materials have been examined it will be possible to compute the extent to which the excess of acid-forming elements in meat and cereal products is offset by that of bases in other food materials of ordinary mixed diets.

Remembering that the 100-calorie portion of any staple food material may be multiplied many times in a day's dietary it is obvious that, by the free use of meats and breadstuffs on the one hand or of fruits, vegetables and milk on the other, the net excess of acid or base introduced into the body through the food may be varied at will within wide limits. It is not our purpose, however, to discuss the possible physiological applications until the data for a larger number of food materials have been obtained.

ON THE EXCRETION OF CREATININ IN THE NEW-BORN INFANT.

(A Preliminary Report.)

By S. AMBERG and W. P. MORRILL.

(*From the Pharmacological Laboratory of the Johns Hopkins University.*)

(Received for publication, June 21, 1907.)

Satisfactory data concerning the metabolism of the new-born are relatively few. Inasmuch as it is very important to know the peculiarities of the metabolism in the new-born infant and in young animals, both from a clinical and from a physiological point of view, we have undertaken investigations in this field with the newer methods and in the light of the newer interpretations of metabolism experiments. The results thus far obtained are presented in their present incomplete state because it was necessary to discontinue the work for some time. However, the results thus far obtained are of sufficient interest to warrant publication at this time.

The investigations of Rietschel[1] concerning the creatinin metabolism of infants led him to the conclusion that the urine of normal infants does not contain any creatinin. In febrile diseases a small amount of creatinin seemed to be excreted. In two cases in which creatinin-zinc chloride was given *per os* the urine gave a strong reaction for creatinin. Reitschel used the creatinin-zinc chloride method to demonstrate the presence or absence of creatinin in the urine. The sediment was examined microscopically and then dissolved in water in order to test for creatinin by the Weyl reaction. No attempt was made to determine quantitatively the creatinin in twenty-four hour specimens of urine. Reitschel mentions that Grocco, an earlier investigator, occasionally found creatinin in very small amounts in the urine

[1] Zur Kentniss des Stoffwechsels beim Saüglinge, *Jahrbuch für Kinderheilkunde*, lxi, p. 615, 1905.

of infants. Von Hoogenhuyze and Verploegh[1] constantly found creatinin in the infants' urine and more distinctly with the reaction of Jaffé than with that of Weyl. In only four cases were exact colorimetric determinations by Folin's method possible on account of the low concentration and small amount of urine obtainable. In these four cases the creatinin content per 10.0 cc. of urine was (1) 1.11 mg., (2) 0.91 mg., (3) 0.41 mg., (4) 1.7 mg. The lowest value (3) was obtained from the urine of a weak, artificially fed baby, while the other babies were vigorous and breast fed.

Closson[2] reports the quantitative determination of creatinin in the case of two boys. The first boy, 6 years and 9 months old and weighing 22 kilos, excreted 0.42 gram and 0.32 gram of creatinin in 24 hours on a meat-free diet.[3] The second boy, a case of diabetes insipidus, 14 years of age and weighing 27.5 kilos eliminated 0.44 gram of creatinin in 24 hours. Furthermore this author demonstrated the presence of creatinin in the urine of young puppies and of kittens.

Our first experiments consisted of qualitative tests for creatinin in specimens of urine from infants; later the creatinin was determined quantitatively according to Folin's method, and in five cases the total creatinin excretion in 24 hours was determined.

In several afebrile cases the Weyl reaction with the fresh urine was negative and the Jaffé reaction doubtful. But while these reactions may be doubtful or negative with 5.0 cc. to 10.0 cc. of fresh urine, the following instance demonstrates that such results may be easily accounted for by the low concentration of the urine. Thus, urine of a strong breast-fed infant did not give a definite reaction when tested directly, but after evaporation of a larger amount of urine and dilution up to 25 per cent of the original volume, both reactions were strongly positive. The dilution in this case was such as to bring the concentration up to about that of normal adult urine. By evaporating the urine or

[1] Von Hoogenhuyze and Verploegh: Beobachtungen über die Kreatinin Ausscheidung beim Menschen, *Zeitschr. f. physiol. Chem.*, xlvi, p. 415, 1905.

[2] Closson: The Elimination of Creatinin, *Amer. Journ. of Physiol.*, xvi, p. 252, 1906.

[3] The creatinin was determined by the Neubauer-Salkowski method.

by using larger amounts of fresh urine, we never failed to get a positive reaction for creatinin.[1]

The quantitative estimations of creatinin were made according to Folin's method[2] using the Duboscq colorimeter for the colorimetric comparison. Since the total amount of creatinin in the infants' urine is small, a larger amount of urine must be used in order to carry out the determination than is required in the case of an adult. Preliminary experiments showed that it is not feasible to work directly with large amounts of urine but that the infants' urine must be concentrated. For instance 5.0 cc. of the urine of an adult gave 22.5 milligrams of creatinin per 10.0 cc. Then 5.0 cc. of this urine were diluted to 100.0 cc. with water and now our first reading showed 20.4 milligrams per 10.0 cc. After standing for 10–12 minutes the readings changed suddenly and corresponded to 15.6 milligrams of creatinin per 10.0 cc. In a second test the same urine diluted as before gave immediately only 13.2 milligrams per 10.0 cc. In a second series 10.0 cc. urine gave 12.3 milligrams creatinin. Then 10.0 cc. of the same urine were diluted to 100.0 cc. with water and after the addition of the picric acid and sodium hydroxide the mixture was allowed to stand 6 minutes before diluting to 500 cc. A second test was allowed to stand 12 minutes and a third 18 minutes. The first test gave 6 milligrams of creatinin, the second 9.3 milligrams, and the third 10.2 milligrams. On account of these results this mode of procedure was abandoned. Then 5.0 cc. of the urine used in the last series were diluted with 95.0 cc. of water, evaporated to dryness on the water bath, the residue taken up in water, filtered and washed with water until the filtrate had a volume of 10.0–12.0 cc. This filtrate was used for a determination, giving a result of 12.4 milligrams, per 10.0 cc. of urine as against 12.3 milligrams as determined directly. In another experiment in which 10.0 cc. of urine were diluted with 90.0 cc. of water and treated in the same manner, the determination gave 12.1 milligrams of creatinin, showing that this method

[1] It is interesting to note that 100.0 cc. of amniotic fluid after removal of the proteid and evaporation to 5.0 cc. gave positive Weyl and Jaffé reactions for creatinin.

[2] Folin: Beitrag zur Chemie des Kreatinins und Kreatins im Harn, *Zeitschr. f. physiol. Chem.*, xli, p. 223, 1904.

is accurate for urine containing small amounts of creatinin. In all the quantitative determinations therefore the urine of the infants was evaporated to dryness on the water bath, taken up in water and filtered in the manner described, the resulting 10.0 cc. of filtrate being used for the colorimetric determination. A further advantage of this method is that it excludes any possibility of error that might arise from the presence of acetone or hydrogen sulphide in the urine. None of the urine used for these experiments contained albumin or reduced Fehling's solution.

The results of the experiments, obtained in three healthy, breast-fed infants 10–14 days old, were as follows:

	Cc. of Urine Used.	Creatinin in Milligrams per 10 cc. of Urine.
1	100.0	1.18
2	77.0	1.56
3	50.0	1.53

Of some interest are the data obtained in several consecutive experiments on an emaciated female child, 2½ years old, suffering from a chronic enteritis probably of tubercular origin. The child died 10 days after the last determination. The urine was obtained by catheterisation. In the first examination the specific gravity was 1005. Seventy-two cc. of urine gave a definite Jaffé reaction but the color was not of sufficient intensity to permit a quantitative determination of the creatinin as the urine was used without previous concentration.

In the following determinations the urine was evaporated and treated as above.

Temp. F.	Weight.		Amt. of Urine Used. cc.	Specific Gravity.	Creatinin per 10 cc. Urine. mg.	Creatin per 10.0 cc. Urine mg.
	lb.	oz.				
99	16	10	30.0		3.9	
98.2	15	7	80.0	1002	0.65	0.11
99.8	14	11	17.5	1010	9.07	
99.8	14	13	62.0	1004	1.87	
102.4	14	9	59.0	1004	2.42	

These results may to a certain extent bear out the observation of Rietschel[1] that fever may influence the creatinin excretion

[1] *Loc. cit.*

and of Leathes,[1] who found an increased output of creatinin in febrile diseases. In our case the creatinin excretion seemed to vary more with the changes in the concentration of the urine than with the changes in the temperature, but since we did not have the 24 hour urine our observations do not permit us to draw any definite conclusions thereto. Similarly the results of Von Hoogenhuyze and Verploegh[2] are of only limited value since they also did not have the 24 hour urine and, while further investigations may confirm their observation that the weak, artificially fed infant excreted less creatinin than the other vigorous breast-fed babies, their results do not justify such a conclusion.

In order to determine the normal daily excretion of creatinin the urine of five male infants was collected for 24 hours. All these infants were inmates of the maternity ward of the Johns Hopkins Hospital, and we desire here to acknowledge the kindness of Professor Williams and of Dr. Goldsborough who placed the necessary material at our disposal. The urine was collected by a modification of the method described by Freund.[3]

Small round flasks of about 180 cc. capacity with a curved neck were attached to the pubic region by means of a rubber collar. The flask was enclosed in a muslin sack and these sacks were again tied around the abdomen, thus affording greater security. This additional security was very desirable since the babies had to be left entirely to the mothers' care during nursings and so were not always carefully handled. The flasks were changed before each nursing in order to avoid any loss which might be incurred in handling the baby. When in bed the legs of the child should be crossed over the flask rather than under it as will happen if they are not watched. This insured the best position of the flask and in this position the babies remained perfectly quiet not requiring to be tied as required by the method of Freund. By this method it was possible to collect 24 hour specimens without disturbing the routine of the nursings, etc., and in some cases in fact the mothers did not know anything unusual was going on. During the time the collection was made, the total amount of food was determined by weighing the child before and after each nursing.

In the following table the age and weight of the infant, the food taken and the urine voided in 24 hours are given, the last

[1] Leathes: On the Excretion of Nitrogen, Creatinin and Uric Acid in Fever, *Journ. of Physiol.*, xxxv, p. 205, 1907.

[2] *Loc. cit.*

[3] Chlor und Stickstoff im Saüglingsorganismus, etc., *Jahrbuch für Kinderheilkunde*, xlviii, p. 137, 1898.

also being given in per cent of total ingestion. The weight of the infant was taken at the end of the 24 hours. In a second table the daily weight from date of birth is recorded in order to give an idea of the general condition of the infants.

No.	Age. Days.	Weight. kilos.	Amt. of Food. gms.	Amt. of Urine. cc.	Amt. of Urine in Per Cent of Food.
1	10	4.033	793.0	320.0	40.4
2	10	3.463	620.0	350.0	56.5
3	14	2.723	252.0	168.0	66.7
4	7	3.093	495.0	200.0	40.4
5	13	3.1	772.0	426.0	55.2

No.	Birth.	1 d.	2 d.	3 d.	4 d.	5 d.	6 d.	7 d.
1	3.715	3.65	3.57	3.625	3.69	3.72	3.812	3.92
2	3.19	3.095	2.985	2.95	3.085	3.15	3.225	3.275
3	2.45	2.35	2.32	2.29	2.265	2.27	2.345	2.39
4	3.12	2.97	2.89	2.95	2.985	3.02	3.065	3.093
5	3.1	3.0	2.842	2.86	2.885	2.87	2.9	2.89

No.	8 d.	9 d.	10 d.	11 d.	12 d.	13 d.	14 d.
1	3.92	3.956	4.033				
2	3.4	3.423	3.463				
3	2.422	2.495	2.535	2.58	2.635	2.	2.723
4							
5	2.947	2.96	2.995	2.98	3.04	3.1	

In the following table the total excretion of nitrogen and of creatinin during 24 hours is given together with the amount of creatinin per kilo of body weight and the per cent of the total nitrogen appearing as creatinin.

No.	Creatinin.		Creatin.		Total N. gms.	Creatinin N Per Cent of Total N.
	Total in 24 hr. mg.	Per K. Body Wt. mg.	Total.	Per K. Body Wt.		
1	26.82	6.71			0.389	2.56
2	26.92	7.78			0.3267	3.06
3	27.05	9.94			0.2785	3.61
4	17.48	5.46			0.218	2.98
5	30.1	9.7	2.87	0.93	0.411	2.72

Besides the determination of the total nitrogen and the creatinin it was our intention to follow the nitrogen distribution, taking into consideration the total nitrogen, ammonia, urea, uric acid, creatinin, and creatin, but since the amounts of urine required for the quantitative determination of these substances far exceeded our supply, we will have to defer the study of the nitrogen distribution to a later publication. It may be mentioned that, with the exception of No. 5, we used 100.0 cc. of urine for the determination of creatinin, 20.0 cc. for ammonia determination (according to Folin[1]), 100.0 cc. evaporated to half the original volume for the urea determination (according to Schöndorf[2]), 20.0 cc. for the total nitrogen, and 150.0 cc. for the uric acid (according to Folin). In the case of No. 5 in which the creatin was determined, only 70.0 cc. were used for the creatinin determination and the uric acid determination was omitted. The results obtained in this case are as follows:

No.	Total N.	Urea N.	Ammonia N.	Creatinin N.
5	0.411 gm.	91.32 per cent.	4.15 per cent.	2.72 per cent.

In order to secure enough urine to make all of these determinations, we tried to collect the urine of two consecutive days, but on the first day we were unable to watch the collection closely enough and the amounts obtained certainly did not represent the 24 hour urine. Since we had to mix the urine of both days in order to carry out the urea and uric acid determinations we will refrain from giving these results until we have further data. The total nitrogen and the creatinin, however, were in every case determined on the urine of the same day. In later experiments we intend to take the determination of the nitrogen in the food into consideration.

To return to our results obtained thus far, No. 4 showed a very low excretion of creatinin and the excretion of total nitrogen too is rather low. Therefore it is highly probable that in this case some urine was lost and we can not consider it as giving normal results. In the other four cases the amount of creatinin

[1] Folin: Approximately Complete Analyses of Thirty "Normal" Urines, *Amer. Jour. of Physiol.*, xiii, p. 45, 1905.

[2] See Emerson: *Clinical Diagnosis*, p. 107, Lippincott, Phila., 1906, and Schöndorff: *Eine Methode der Harnstoffbestimmung*, etc.

excreted in 24 hours showed remarkably little variation for the different infants (26.82–30.1 milligrams) although there was considerable variation in the total quantity of urine and therefore in the creatinin concentration. Furthermore, considering the excretion of creatinin per kilo of body weight, we see that No. 1 presents the lowest value, while No. 3 presents the highest. No. 1 was a very fat baby while No. 3 was not very well nourished, thus showing a close agreement to the statement of Folin[1] that fat or corpulent persons yield less creatinin per unit body weight than lean ones.

There is a very considerable difference between the amount of creatinin excreted by the infant and that excreted by the adult. The values for the latter vary according to Folin between 20 and 24 milligrams per kilo of body weight depending on the corpulency of the individual. Shaffer[2] more recently found variations from 18.0 to 30.0 milligrams per kilo for the normal adult. In our cases the elimination of creatinin per kilo varied between 6.71 and 9.94 milligrams and since moderate muscular exercise does not influence the creatinin elimination (see Folin,[3] Von Hoogenhuzye and Verploegh[4] and Otto of Klercker[5]) the lack of it can not be responsible for these low values. Benedict and Myers[6] studied the creatinin excretion in women in order to throw additional light on the three factors which have been claimed to influence the creatinin elimination, namely, the degree of muscular development, the muscle tonus (Shaffer), and the proportion of adipose tissue. Of interest are their observations that in general the creatinin excretion of women is much lower than that of men, and in two cases of advanced age the creatinin coefficient, that is, the creatinin elimination in milligrams per kilo body weight, fell to 7.4. In general though they found the creatinin excretion proportional to the body weight and not to

[1] Folin: Laws Governing the Chemical Composition of the Urine, *Amer. Journ. of Physiol.*, xiii, p. 85, 1905.

[2] Cited by Benedict and Myers.

[3] *Amer. Journ. of Physiol.*, xiii, p. 85, 1905.

[4] *Loc. cit.*

[5] Otto of Klercker: Kreatin and Kreatinin im Stoffwechsel, *Biochem. Zeitschr.*, iii, p. 33, 1907.

[6] Benedict and Myers: The Elimination of Creatinin in Women, *Amer. Journ. of Physiol.*, xviii, p. 377, 1907.

the active mass of protoplasmic tissue, thus denying the influence of inert adipose tissue. Furthermore, they were unable to show a constant proportion between muscular development or muscle tonus and the creatinin elimination. We can not enter here into a discussion of these conclusions, but it appears that they will have to be supported by further investigations.

Our results appear to speak in favor of the theory advanced by Folin and also for that of Shaffer, at least in so far as muscular development is concerned. The body of the new-born child[1] is relatively richer in water and fat, while poorer in nitrogenous substance and ash than the body of the adult, and the muscles of the new-born child constitute only 23 per cent of its total body weight, while those of an adult constitute 43 per cent of the total weight.

Another point must be mentioned. The diet of our babies was one which, in the light of Folin's studies, is rich in proteids. This we may deduce from the relative amount of creatinin nitrogen which represents an average of 2.99 per cent of the total nitrogen. The proteid-rich diet yielded, as cited by Folin and taken from one of his tables, 3.6 per cent creatinin nitrogen, while the diet poor in proteid yielded 17.2 per cent. The relatively high percentage of urea nitrogen in our cases would point in the same direction. In accordance with Folin's views then the end products of the metabolism of the normal breast-fed infant would demonstrate a pronounced exogenous nitrogen metabolism; in other words, we would have an indication of a "Luxus" consumption of proteid. Whether such a diet rich in proteid is warranted by the demands of growth is a question which can not be discussed on the basis of our experiments. From the exposition of Camerer, however, it is doubtful that such is the case.

In this discussion we have followed the theory of Folin[2] that in the creatinin excretion we have one of the most important indicators of the endogenous nitrogen metabolism, or tissue metabolism while only a small portion of the urea could be derived

[1] Camerer in Pfaundler und Schlossman: *Handbuch der Kinderheilkunde*. Leipzig, 1906. Stoffwechsel und Ernährung im Ersten Lebenjahr.

[2] Folin: A Theory of Proteid Metabolism, *Amer. Journ. of Physiol.*, xiii, p. 117, 1905.

from that source, though we are well aware that this conception is not generally accepted. Thus Leathes[1] concludes that the urea constitutes the principal nitrogenous product of tissue metabolism. Therefore we will have to be extremely cautious in concluding from our experiments that we are actually confronted by a "Luxus" consumption of proteid.

SUMMARY.

1. Creatinin is constantly present in the urine of normal breast-fed infants.

2. The creatinin coefficient while only approximately one-third as great as in adults is fully as constant.

3. Taking into consideration the relative percentages of muscle substance in the adult and in the infant, we are inclined to favor the theory that the creatinin excretion is an important index of endogenous proteid metabolism.

[1] *Loc. cit.*

THE EFFECT OF TRANSFUSION OF BLOOD ON THE NITROGENOUS METABOLISM OF DOGS.

By HOWARD D. HASKINS.[1]

(*From the Physiological Laboratory, Western Reserve University.*)

(Received for publication, June 5, 1907.)

The subjects of the experiments reported in this paper were healthy small dogs.

The operations for transfusion were performed by Dr. George W. Crile and his assistants, Drs. Lenhart and Hitchings. The surgical aspects of transfusion will be fully considered by Dr. Crile in other journals.

METHODS: The dogs were put on to a uniform diet, consisting of milk (230–265 cc.) and dog biscuit, the amount given being proportionate to the body weight. A quantity of the dog biscuit sufficient for all the experiments was ground up, and after being thoroughly mixed was kept in a large bottle. Analysis of samples of this showed 3.3 per cent of nitrogen.

It will be observed from the tables that the dogs were practically in nitrogenous equilibrium before the operations took place. The dogs were kept in a large metabolism cage and were catheterized every 24 hours.

As to the methods of analysis of the urine, Kjeldahl's method was used for total nitrogen, while Folin's methods[2] were used for estimating urea, ammonia, uric acid and creatinin. Inasmuch as I did not have access to a suitable apparatus, Dr. Folin himself kindly made the creatinin estimations for me.

CONSIDERATION OF RESULTS. *Experiment 1:* Dog A (see Table I) was put on the milk and biscuit diet until the nitrogenous excretion had become constant, when a control operation was performed. In this, the dog was kept under ether for one hour, during which time the femoral blood vessels were exposed but

[1] H. M. Hanna Research Fellow, Western Reserve University.

[2] Folin: *Amer. Journ. of Physiol.*, xiii, p. 45, 1905.

not injured. This operation resulted in a slight increase in the excretion of total nitrogen and some increase of that of ammonia. A few days later a transfusion was performed, 250 cc. of blood being withdrawn and being replaced immediately afterwards by 230 cc. from Dog B. The latter dog had not been given the preparatory diet, but was taken from the dog room to replace the dog that had been specially dieted for this purpose but was accidentally killed. Even after allowing for body weight and for the effect of hemorrhage, Dog B was shown, by the urines following operation (Table II) to be on a distinctly higher plane of excretion than Dog A as regards total nitrogen, ammonia, uric acid and creatinin. There was a marked change in the composition of the urine of Dog A after transfusion; namely, an increase affecting total nitrogen and urea.

We might suppose that this change was due to the character of the blood received. To decide this it was necessary to compare the above effect with that of hemorrhage alone. When the wounds had healed sufficiently and the composition of the urine had come back almost to what it was before transfusion, another operation was performed in which 130 cc. of blood were removed.

On comparing the effect of hemorrhage (+ anesthesia) with the effect of transfusion alone in the same dog the following are noted:

As regards nitrogen excretion, averaging two days before and two after operation, hemorrhage caused 14.7 per cent increase, transfusion 20 per cent; as regards the per cent of nitrogen present as ammonia, averaging four days before and after, hemorrhage caused an increase amounting to 11 per cent, transfusion 15 per cent; as regards the per cent of nitrogen excreted as urea, a more marked decrease occurred from hemorrhage alone—dropping from 85.7 per cent to 72 per cent—than from transfusion, which caused a drop from 84.2 per cent to 76.8 per cent. Uric acid was increased for one day in both cases, being 18 per cent higher after transfusion and 14 per cent after hemorrhage, as compared with the day before operation. Creatinin decreased 7.8 per cent after hemorrhage, and increased 3.7 per cent (averaging several estimations before and after) after transfusion. This increase of creatinin does not seem to be at all proportional to the very high excretion of the donor (Dog B).

TABLE I. DOG A.

Urine No.	Date, 1907.	Volume of Urine, cc.	Nitrogen in Urine, grams.	Urea Nitrogen, grams.	Ammonia Nitrogen, grams.	In Per Cent of Total Nitrogen.		Uric Acid, grams.	Creatinin, grams.	Remarks.
						Urea N	NH_3N			
1	Jan. 14	123	3.577		.182		5.0			Milk and biscuit diet.
3	" 16	145	2.037		.102		5.0			
5	" 18	135	2.226		.051		2.2			
9	" 22	195	2.835		.134		4.7			
11	" 24	140	2.254		.108		4.8			Weight, 6.6 kg.
13	" 27	217	2.317	1.884	.062	81.3	2.6	.020		2.2 gm. nitrogen in food.
14	" 29	290	2.461	2.075	.067	84.3	2.7	.020	.171	
15	" 30	340	2.730	2.246	.078	82.2	2.8	.027	.167	Weight, 6.5 kg.
16	" 31	160	2.051		.056		2.7	.020		
17	Feb. 1	135	2.107	1.791	.050	85.0	2.4	.020	.150	Control operation, anesthesia 1 hr.
18	" 2	150	2.506		.075		3.0		.165	
19	" 3	150	2.352	1.989	.098	84.2	4.1	.022	.150	
	" 4									Transfusion; 250 cc. blood drawn; 230 cc. blood transfused from B.
20	" 5	168	3.160	2.428	.071	76.8	2.2	.026	.162	
21	" 6	100	2.695	2.114	.112	78.3	4.1		.160	
22	" 7	140	2.905	2.625	.112	90.5	3.8	.019	.175	Weight, 6.1 kg.
23	" 8	145	2.765	2.124	.137	76.8	4.9	.020	.170	Wound suppurating.
26	" 13	175	2.730		.066		2.4	.018		Wound healthy, 2.5 gm. nitrogen in food
28	" 16	154	2.807		.083		2.9	.022		
29	" 18	285	3.724	3.184	.134	85.0	3.6	.025	.160	Weight, 5.7 kg.
30	" 19	200	3.250	2.788	.043	85.7	1.3	.028	.144	
	" 19									Operation—hemorrhage 130 cc. blood drawn.
31	" 20	120	3.591	2.891	.070	72.0	1.9	.032	.140	
32	" 21	181	4.410	3.275	.106	74.2	2.4	.028	.142	
33	" 22	105	3.220		.104		3.2			Wound healthy.
34	" 23	125	2.780	2.313	.109	71.8	3.9		.136	
36	" 27	160	2.597		.087		3.3	.023	.142	Weight, 5.6 kg.

The effect of hemorrhage in increasing total nitrogen excretion had been previously observed by Hawk and Gies.[1]

Experiment 2: In this case both dogs (see Tables III and IV) were under exactly the same dietary conditions and their excretions were just about the same, when allowance is made for the difference in body weight. The amount of blood drawn and transfused was not determined.

Unfortunately the effect of hemorrhage on the excretion of Dog D could not be studied very successfully because accidents prevented the collecting of the urine for two days after the operation.

With Dog C, transfusion caused 35 per cent increase of nitrogen (averaging two days before and after operation), 48 per cent increase of ammonia nitrogen per cent (averaging three days before and after), 22 per cent increase of uric acid, and 6.5 per cent increase of creatinin (averaging two days before and after).

By comparing with Experiment 1, we find that the increases are greater in the case of C than in that of A. Therefore, we cannot argue that the blood of the higher (meat) diet dog, B, produced any characteristic effect different from that produced by the blood of the medium diet (milk and biscuit) dog, D. Diet does not seem to be an essential factor. Judging from the results in these two experiments, it would seem that transfusion (or rather hemorrhage followed by transfusion) produces practically the same effect on nitrogenous metabolism as does moderate hemorrhage alone, but to a much greater degree, and further, that the nature of the diet given the donor previous to transfusion is of no importance in influencing the result.

A third transfusion was successfully performed, but unfortunately the dog refused food and was quite sick following the operation, so that further work on the urines was rendered valueless.

In the case of all the other dogs, transfusion and hemorrhage were quickly recovered from, in so far as could be judged by their appearance and behavior.

The work reported here is hardly extensive enough to warrant

[1] Hawk and Gies: The Influence of External Hemorrhage. *Amer. Journ. of Physiol.*, xi, p. 171.

TABLE II. DOG B.

Urine No.	Date, 1907.	Volume of Urine, cc.	Nitrogen in Urine, grams.	Urea Nitrogen, grams.	Ammonia Nitrogen, grams.	In Per Cent of Total Nitrogen.		Uric Acid, grams.	Creatinin, grams.	Remarks.
						Urea N	NH_3N			
1	Feb. 5		6.818	5.754	.308	81.0	4.5			On meat diet before operation; lost 250 cc. blood transfused into A.
2	" 6		4.774		.252		5.2			Weight, 13 kg.

TABLE III. DOG C.

Urine No.	Date, 1907.	Volume of Urine, cc.	Nitrogen in Urine, grams.	Ammonia Nitrogen, in grams.	In Per Cent of Total Nitrogen	Uric Acid, grams.	Creatinin in grams.	Remarks.
1	March 2		2.842	.087	3.0		.175	Has been on milk and biscuit diet several days; 2.73 gm. nitrogen in food.
2	" 4	190	2.310	.073	3.1	.026	.143	Weight, 6.5 kg.
3	" 5	165	2.492	.073	1.6	.027	.146	
	" 5							Operation—hemorrhage followed by transfusion from D.
4	" 6	170	3.268	.056	1.7	.033	.153	
5	" 7	200	3.255	.142	4.3	.024	.155	
6	" 8		2.328	.138	5.9			
7	" 9	85	2.247	.078	3.4	.021	.139	
8	" 11	140	2.527	.083	3.2			

TABLE IV. DOG D.

1	March 2	65	2.380	.048	2.0	.019	.148	Has been on milk and biscuit diet several days; 2.47 gm. nitrogen in food.
2	" 5	105	2.156	.108	5.0	.109	.130	Weight, 5.5 kg.
3	" 5							Operation—Gave blood transfused into C.
4	" 8	112	2.520	.073	2.8	.020	.154	Could not collect urine for several days.
	" 9	76	2.24	.095	4.2	.024	.146	

the drawing of any conclusions further than that transfusion of blood following hemorrhage has practically the same effect on nitrogenous metabolism as hemorrhage alone has. In other words, the transfusion of normal blood after hemorrhage does not prevent the effect on nitrogenous metabolism of hemorrhage alone.

THE PICROLONATES OF CERTAIN ALKALOIDS.

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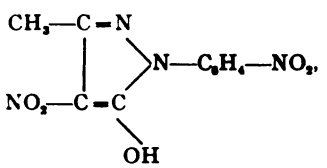
(Plates III-VIII.)

Our attention was called to the use of picrolonic acid in precipitating alkaloids and we have studied its behavior with some of the more important representatives of this group. Like picric acid picrolonic acid precipitates many basic nitrogen compounds and, since the latter has given better results in other instances, we thought it advisable to determine the relative value of these reagents as alkaloidal precipitants. They are widely different as far as ease of obtaining them is concerned. Picric acid is cheap and quickly prepared, whereas picrolonic acid is not listed by manufacturers and its preparation is rather difficult.

Knorr[1] gave the name "*picrolonic acid*" to 1-p-nitro-phenyl-3-methyl-4-nitro-5-pyrazolon,



which he preferred to picric acid in studying 1,2-ethanol-amine and certain morpholine derivatives, because of the greater insolubility of their picrolonates and the ease with which these could be recrystallized and identified. Recently Steudel[2] has pointed out the value of this reagent in isolating and identifying the two hexone bases, arginin and histidin.

[1] *Ber. d. deutsch. chem. Gesellsch.*, xxx, p. 909, and xxxii, pp. 732 and 736.
[2] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 219.

PREPARATION OF PICROLONIC ACID.

Methyl-phenyl-pyrazolon, prepared from acetacetic ester and phenylhydrazine, according to Knorr,[1] was converted into picrolonic acid by Bertram's method as described by Steudel.[2] In the hope of improving the yield we tried several modifications of this method but had indifferent success. Finally we employed the method of Braun[3] which gave a result so much better than any we had obtained up to that time that we can recommend it for the preparation of picrolonic acid. As this method is somewhat inaccessible, we have described our manner of carrying it out.

Six hundred cc. of 90 per cent nitric acid (sp. gr., 1.49), prepared by diluting 540 cc. of strongest fuming nitric acid (sp. gr., 1.52 = 99.7 per cent) with 60 cc. of water, were kept cold with ice-water in a wide-mouth bottle. Two hundred grams of methyl-phenyl-pyrazolon were added gram at a time, the mixture being mechanically stirred all the while and the temperature being kept below 15° C. Each addition of substance caused a vigorous reaction attended with escape of red fumes. Halfway in the process, a reddish-yellow, crystalline product began to appear. At the end, the mixture was stirred for thirty minutes and then the crystalline mass was collected upon a small porcelain disc covered with asbestos, freed from acid by suction and washed successively with dilute nitric acid and with water. As this crude product gradually undergoes decomposition, it is well to proceed at once with the next step in the process.

Fifteen hundred cc. of 33 per cent acetic acid, about six times the weight of the crude product, were mechanically stirred in a beaker on the water-bath. The crude product finely ground was added and the temperature was not allowed to exceed 60° C. This substance, which is heavy and granular, tends to settle to the bottom of the beaker. At low temperatures it remains unaltered but gradually changes as the temperature rises. The change is rapid from 50° to 60°. A mass of light-yellow, flocculent crystals replaces the reddish-yellow compound and fills the liquid. Saponification is complete in 15 to 40 minutes. The product may be examined occasionally under the microscope, if necessary, and the reaction is complete when only needles of picrolonic acid are to be seen.

The crude picrolonic acid, freed from acid on a Büchner filter, was washed with water and then ground in a mortar with 150 grams of crystallized sodium carbonate. Carbon dioxide was given off and the yellow sodium salt was formed. This was pressed out and crystallized from a

[1] *Ber. d. deutsch. chem. Gesellsch.*, xvi, p. 2597.

[2] *Zeitschr. f. physiol. Chem.*, xliv, p. 157.

[3] Inaugural Dissertation, Jena, 1899.

mixture of three volumes of strong alcohol and one volume of water. Sodium picrolonate was then decomposed with concentrated hydrochloric acid. The acid was warmed in a beaker on the water-bath, stirred as before and the sodium salt was added in small portions. Picrolonic acid separated as a fine, mealy powder. It was thrown on a porcelain disc covered with asbestos, freed from acid by suction and washed with water to remove acid and sodium chloride. We obtained 166 grams of picrolonic acid by this method from 200 grams of methyl-phenyl-pyrazolon. If further purification is desired, picrolonic acid may be crystallized from strong alcohol from which it separates in fine yellow needles which melt at 116.5° C.

USE OF PICROLONIC ACID.

A saturated alcoholic solution of picrolonic acid is the most convenient form in which to use this reagent for ordinary purposes. Such a solution may be employed to precipitate alkaloids from water, alcohol or from other solvents miscible with alcohol. Although picrolonic acid itself is only slightly soluble in water, sufficient alcohol is present to prevent the reagent from being precipitated when added in excess to aqueous solutions.

In some cases it may be an advantage, after extracting an alkaloid from an aqueous, alkaline solution with such solvents as ether, chloroform, benzol, acetic ether and amyl alcohol, to substitute for the alcoholic reagent a saturated solution of picrolonic acid in the particular solvent used to extract the alkaloid. In isolating morphine, for example, the alkaline solution may be extracted either with hot chloroform or with hot amyl alcohol and this extract may be tested directly with a solution of picrolonic acid in the corresponding solvent. The following table shows the relative solubility of picrolonic acid in the solvents mentioned above:

SOLUBILITIES OF PICROLONIC ACID AT ORDINARY TEMPERATURE.

Solvent.	Acid in 1 cc. in Grams.
Water	0.0017
Alcohol	0.0209
Ether	0.005
Benzol	0.0024
Chloroform	0.015
Acetic ether	0.041
Amyl alcohol	0.0056

To recover an alkaloid from its picrolonate, it is necessary only to warm the latter with dilute sulphuric acid which discharges the bright yellow color of the picrolonate, causing the alkaloid to pass into solution and precipitating pale-yellow picrolonic acid. By extracting with acetic ether, in which picrolonic acid is especially soluble, the aqueous solution of the alkaloid is left colorless. This has suggested a method of purifying alkaloids after their extraction in the usual way from animal tissues. An alkaloid like strychnine, whose picrolonate is very insoluble, may be precipitated from aqueous solution and thus separated from other substances which interfere with its purification. The precipitated picrolonate may be collected on a filter, washed with water and then warmed with dilute sulphuric acid. After extracting the free picrolonic acid as before, a very pure solution of the alkaloid will remain.

COMPARISON OF PICROLONIC ACID AND PICRIC ACID.

Although picric acid may be used either in aqueous or in alcoholic solution, we were limited in the case of picrolonic acid to alcohol because of the slight solubility of this substance in water. The two reagents were prepared by saturating 90 per cent alcohol with these acids. Such a solution of picric acid contains at ordinary temperature 0.0596 gram in 1 cc. and the corresponding solution of picrolonic acid contains 0.0209 gram in 1 cc. There is no precipitation of either reagent upon adding it to 5 cc. of water volume for volume, or in smaller quantity. A precipitate, therefore, in an actual test is due to alkaloid and not to dilution of the reagent with water.

The solution of each alkaloid was prepared either by dissolving one of its salts in water, or by dissolving the alkaloid itself in water with the aid of dilute acetic acid. We began in each case with a 1 per cent solution, aliquot volumes of which were diluted with water until a point was reached where the test failed. Time is a factor to be considered in deciding whether a test is positive or negative, especially when the solutions are dilute. The precipitate may not be immediate but it will sometimes appear upon standing. We adopted fifteen minutes as an arbitrary limit and called the test negative when there was no

precipitate within that time. Frequently there was a turbidity instead of a distinct precipitate but this sometimes became crystalline upon standing. We decided to accept as positive those tests where a turbidity gave such a result. The relative delicacy of the two reagents toward the alkaloids studied appears in the following table:

DELICACY OF REAGENTS AS ALKALOIDAL PRECIPITANTS.

Alkaloids.	Picric Acid.		Picrolonic Acid.	
	Positive in 15 min.	Remarks.	Positive in 15 min.	Remarks.
Coniine.....	1-250	Turbidity without precipitate	1-800	Very slight in 15 min.
Nicotine.....	1-20000	Slight crystalline precipitate	1-20000	Slight crystalline precipitate
Strychnine....	1-40000	Very slight in 15 min.	1-75000	Very slight in 15 min.
Brucine.....	1-20000	Positive in 12 min.	1-10000	Very slight in 15 min.
Morphine.....	1-400	Very slight in 15 min.	1-500	Positive in 30 min. in 1-1000
Codeine.....	1-1000	Very slight	1-500	Very slight
Atropine.....	1-1000	Turbidity which becomes crystalline	1-1000	Turbidity which becomes crystalline
Quinine.....	1-50000	Turbidity without precipitate	1-50000	Turbidity without precipitate
Hydrastine....	1-10000	Turbidity without precipitate	1-10000	Turbidity which becomes crystalline

Our results show considerable variation in the limit of dilution within which alkaloids can be detected with these reagents. Strychnine, which can be detected by picrolonic acid in 1-75000, affords the most delicate test, whereas coniine can barely be detected by picric acid in 1-250. Yet the difference between these reagents is not as marked as we had expected when we began our experiments. Upon the whole picrolonic acid is the

more sensitive, especially toward coniine, strychnine and morphine. On the other hand, picric acid is the more sensitive toward brucine and codeine. They appear about equally sensitive toward nicotine, quinine, atropine and hydrastine. As a matter of fact picric acid is a better reagent for nicotine, because the precipitate is distinctly crystalline and quite characteristic in 1–20000. This reagent would seem to be well-adapted for detecting this particular alkaloid.

The recognition of these alkaloidal picrolonates and picrates by means of the microscope depends entirely upon the method of preparing these compounds. It is difficult, if not impossible, to distinguish most of these substances when they have been precipitated from aqueous solution. We should hardly expect it to be otherwise. We observed a difference in those cases where there was a turbidity which became crystalline upon standing. The crystalline form was more distinct. For this reason we have always redissolved the substance, preferably in alcohol, and allowed crystallization to take place slowly. This applies more particularly to our later work where we had occasion to examine crystals carefully either to describe them or to take photo-micrographs. Since our results with alkaloidal picrates are for the most part like those of Popoff,[1] who has made an extensive study of picric acid as an alkaloidal precipitant, we have described and photographed only a few of the more important of these compounds.

PROPERTIES OF ALKALOIDAL PICROLONATES AND ANALYTICAL RESULTS.

In preparing alkaloidal picrolonates for analysis and for observing their properties, we have obtained the best results by adding an excess of alcoholic reagent to a hot solution of the free alkaloid in as small a quantity of alcohol as possible. Some picrolonates appear immediately; others crystallize as the solution cools and yet others require long standing or concentration of the solution. Before being analyzed, these compounds were recrystallized at least once and dried at 100–110° C., or by standing in desiccator over sulphuric acid.

[1] *Le Laboratoire de Toxicologie*: Brouardel et Ogier, pp. 203–220, 1891.

Coniine Picrolonate—$C_8H_{17}N.C_{10}H_8N_4O_5$.

Coniine picrolonate was prepared by adding a slight excess of reagent to Merck's pure coniine dissolved in a small quantity of alcohol. Since the precipitate, which appeared immediately, did not show the characteristic crystalline form of this substance, it was recrystallized once from alcohol somewhat diluted with water. Coniine picrolonate forms large rhombohedrons when it crystallizes slowly from alcohol, but when crystallization is rapid it appears as short, thick, irregular crystals with pointed ends and these are often in rosettes (Fig. 1). These crystals are quite different from those of the corresponding nicotine compound and serve to distinguish these two liquid alkaloids. Coniine picrolonate is light-yellow, it dissolves readily with heat either in water or in alcohol and melts at 195.5° at which point it becomes dark owing to decomposition. Upon analysis it gave the following result:

0.2252 gram of substance gave 35.5 cc. of nitrogen gas at 21° and 748 mm.

	Calculated for $C_{18}H_{25}N_5O_5$:	Found:
N.....	17.9 per cent.	17.7 per cent.

Nicotine Picrolonate—$C_{10}H_{14}N_2.C_{10}H_8N_4O_5$.

As our available alkaloid showed signs of change due probably to long standing, we prepared the dioxalate and recrystallized this compound until it melted at 110° C. Nicotine picrolonate was precipitated by adding an excess of reagent to an aqueous solution of the dioxalate. The precipitate was crystallized from alcohol somewhat diluted with water. This compound forms long, thin, prismatic needles with square ends which are often grouped in fan-shaped clusters (Fig. 2). It is light-yellow and melts at 213° previously subliming somewhat and turning dark. Upon analysis it gave the following result:

0.2015 gram of substance gave 35.6 cc. of nitrogen gas at 19° and 747 mm.

	Calculated for $C_{20}H_{23}N_6O_5$:	Found:
N.....	19.72 per cent.	19.96 per cent.

Nicotine, though a diacid base, appears to combine with but one molecule of picrolonic acid. On the other hand it combines with two molecules of picric acid. This compound has been

described by Pinner and Wolfenstein[1] and, as stated elsewhere, we have found it preferable to the picrolonate. Aqueous picric acid precipitates nicotine picrate as small yellow needles which melt at 218° and dissolve with difficulty. Crystallized from hot alcohol it appears in short, hexagonal prisms often terminated by hexagonal pyramids (Fig. 3). Some forms resemble quartz crystals.

Strychnine Picrolonate—$C_{21}H_{22}N_2O_2.C_{10}H_8N_4O_5$.

The picrolonates of the two nux vomica alkaloids, strychnine and brucine, melt with difficulty, or not at all, and differ mainly in crystalline form. Strychnine picrolonate was prepared by adding the reagent in excess to a hot alcoholic solution of the alkaloid. The precipitate consists of crystalline leaflets but, when crystallized slowly from alcohol, it appears in rectangular prisms, more or less irregular in form, which are sometimes arranged in star-shaped clusters (Fig. 4). This compound is light-yellow and very insoluble in water or in alcohol of any strength. When heated it begins to darken at 256° and the color increases as the temperature rises, making it difficult to determine the precise melting-point which seemed to be about 275°. Upon analysis it gave the following result:

0.2016 gram of substance gave 24.9 cc. of nitrogen gas at 20° and 747 mm.

	Calculated for $C_{31}H_{30}N_8O_7$:	Found:
N.....	14.04 per cent.	13.9 per cent.

In comparing strychnine picrolonate with the picrate, we found the latter compound mentioned by Guareschi[2] who refers to Gerock's use of the picrates of strychnine and brucine in separating these alkaloids but does not give their properties. Strychnine picrate, prepared by adding an excess of alcoholic reagent to a hot alcoholic solution of the alkaloid, separates from solution in leaflets. Redissolved in alcohol and allowed to crystallize slowly, it appears in short, slightly thickened trapezoidal plates, the non-parallel ends of which are usually irregular

[1] *Ber. d. deutsch. chem. Gesellsch.*, xxiv, p. 66.

[2] *Einführung in das Studium der Alkaloide*, p. 505, 1896.

in outline (Fig. 5). It undergoes decomposition when heated and up to 300° does not melt.

Brucine Picrolonate—$C_{23}H_{26}N_2O_4.C_{10}H_8N_4O_5$.

Brucine picrolonate was prepared by adding the reagent in excess to an alcoholic solution of the alkaloid. The precipitate, which is almost immediate, consists of cubical crystals many of which look like rhombohedrons (Fig. 6). This compound is very insoluble in water or in alcohol of any strength. When heated it begins to darken at 234° and finally melts at 256°. Upon analysis it gave the following result:

0.2011 gram of substance gave 22.8 cc. of nitrogen gas at 20° and 753 mm.

	Calculated for $C_{33}H_{34}N_6O_9$:	Found:
N.....	12.7 per cent.	12.8 per cent.

In comparing brucine picrolonate with the picrate, we noticed a peculiarity in the behavior of the latter. An alcoholic solution of brucine was added to an alcoholic solution of picric acid. There was an immediate yellow crystalline precipitate and, when this was allowed to stand without being filtered, an orange-red compound also appeared gradually. Upon heating the solution to boiling, the yellow substance was dissolved and, as the solution cooled, only the orange-red compound reappeared. This latter substance crystallizes in cubical crystals and in elongated, rectangular prisms terminated by low pyramids (Fig. 7). When heated up to 310°, neither the yellow nor the orange-red compound melts though both undergo decomposition. At 213° the yellow compound becomes orange-red. Judged by the following analyses, there is not a wide difference in their composition:

Yellow Compound—$C_{23}H_{26}N_2O_4.C_6H_2(NO_2)_3OH$.

0.2007 gram of substance gave 19.8 cc. of nitrogen gas at 18° and 739 mm.

	Calculated for $C_{29}H_{29}N_5O_{11}$:	Found:
N.....	11.23 per cent.	11.08 per cent.

Orange-red Compound—$C_{23}H_{26}N_2O_4.C_6H_2(NO_2)_6OH$.

0.2016 gram of substance gave 20 cc. of nitrogen gas at 19° and 751 mm.

	Calculated for $C_{29}H_{29}N_5O_{11}$:	Found:
N.....	11.23 per cent.	11.32 per cent.

Morphine Picrolonate—$C_{17}H_{19}NO_3.C_{10}H_8N_4O_5$.

This compound was prepared by adding the reagent in excess to an alcoholic solution of the alkaloid and evaporating the solution until crystallization began. Morphine picrolonate forms broad, flattened needles which resemble knife-blades and are sometimes arranged in fan-shaped clusters (Fig. 8). It is quite readily soluble in alcohol. When heated it melts at 186.5° and turns dark-brown near its melting-point. Upon analysis it gave the following result:

0.2024 gram of substance gave 22 cc. of nitrogen gas at 16° and 749 mm.

	Calculated for $C_{27}H_{27}N_5O_8$:	Found:
N.....	12.7 per cent.	12.5 per cent.

Codeine Picrolonate—$C_{18}H_{21}NO_3.C_{10}H_8N_4O_5$.

This compound was prepared by adding the reagent in excess to an alcoholic solution of the alkaloid. When the solution is very concentrated and crystallization is rapid, codeine picrolonate appears in short, thick crystals which form rosettes; but when it crystallizes slowly from hot alcohol it forms characteristic crystals looking like truncated pyramids (Fig. 9). It is deep-yellow and melts at 219°, turning orange-red just before melting and becoming dark as it melts. Upon analysis it gave the following result:

0.2054 gram of substance gave 22.4 cc. of nitrogen gas at 20° and 750 mm.

	Calculated for $C_{28}H_{29}N_5O_8$:	Found:
N.....	12.4 per cent.	12.3 per cent.

Atropine Picrolonate—$C_{17}H_{23}NO_3.C_{10}H_8N_4O_5$.

This compound was prepared by adding the reagent in excess to an alcoholic solution of the alkaloid. The yellow precipitate was recrystallized from alcohol in which atropine picrolonate is readily soluble. It appears as short, pointed crystals which are grouped in thick, spherical masses (Fig. 10). It melts at 194° and turns orange-red. Upon analysis it gave the following result:

0.2009 gram of substance gave 22.4 cc. of nitrogen gas at 20° and 752 mm.

	Calculated for $C_{27}H_{31}N_5O_8$:	Found:
N.....	12.66 per cent.	12.63 per cent.

Quinine Picrolonate—$C_{20}H_{24}N_2O_2 . 2\ C_{10}H_8N_4O_5$.

This compound was prepared by adding a hot alcoholic solution of quinine to a hot alcoholic solution of picrolonic acid containing the reagent in the proportion of two molecules to one molecule of the alkaloid. The picrolonate crystallized slowly and seemed rather soluble in alcohol, but in recrystallizing it from hot alcohol we found that a much larger volume of solvent was required for complete solution. This compound appears in hair-like needles which form spherical masses or sheaf-like clusters (Fig. 11). It begins to darken at 223° and melts at 225°. Upon analysis it gave the following result:

0.2007 gram of substance gave 28.8 cc. of nitrogen gas at 19° and 750 mm.

	Calculated for $C_{40}H_{40}N_{10}O_{12}$:	Found:
N.....	16.4 per cent.	16.28 per cent.

Hydrastine Picrolonate—$C_{21}H_{21}NO_6 . C_{10}H_8N_4O_5$.

This compound was prepared by adding the reagent in excess to a hot alcoholic solution of the alkaloid. There was an immediate crystalline precipitate which was recrystallized from hot alcohol somewhat diluted with water. Hydrastine picrolonate is light-yellow and melts at 220°. It appears as long, flat, prismatic needles with square or pointed ends and these are sometimes in spherical clusters (Fig. 12). Upon analysis it gave the following result:

0.2013 gram of substance gave 19.7 cc. of nitrogen gas at 21° and 747 mm.

	Calculated for $C_{31}H_{29}N_5O_{11}$:	Found:
N.....	10.81 per cent.	10.95 per cent.

Other Alkaloids and Picrolonic Acid.

In addition to the preceding nine alkaloids, which form definite crystalline compounds with picrolonic acid, we have also studied the action of this reagent upon cocaine, aconitine and caffeine. We did not succeed in obtaining as satisfactory results with these three alkaloids. Hager[1] found that a saturated aqueous solution of picric acid would not precipitate caffeine and there was some doubt about its giving a precipitate with aconitine.

[1] *Zeitschr. f. anal. Chem.*, ix, p. 110.

Pure cocaine hydrochloride, dissolved in water and treated with alcoholic picrolonic acid, gave only a thick resinous precipitate which did not show the least sign of becoming crystalline after long standing, or when dissolved in alcohol in which it is readily soluble. The same result was obtained when the alkaloid itself was dissolved in alcohol and treated with the reagent. Consequently we did not make any further examination of this substance.

We had a hope that aconitine, for which the number of well-defined tests is limited, might give a characteristic result with picrolonic acid. When pure crystallized aconitine was dissolved in alcohol and treated with picrolonic acid, there was not an immediate precipitate. As the solution evaporated, crystallization began but it was evident that these crystals were not homogeneous. Some of these were yellow and others were white. A result quite similar to this was given also by caffeine. We did not, therefore, continue the examination of the picrolonates of these alkaloids.

In conclusion we desire to acknowledge our obligations to Prof. H. Steudel who directed our attention to the subject considered in these pages and very kindly supplied us with a portion of the picrolonic acid required for the work.

Fig. 1. Coniine Picrolonate.

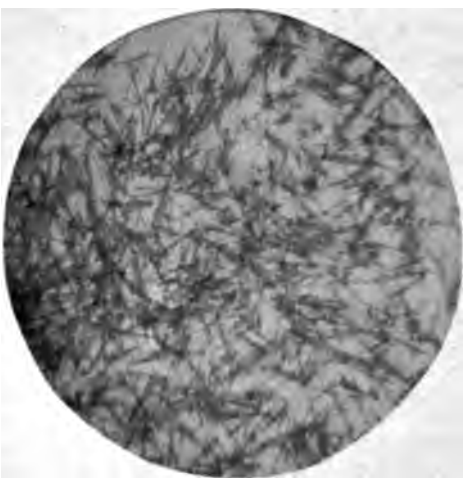


Fig. 2. Nicotine Picrolonate.

Fig. 3. Nicotine Picrate.

Fig. 4. Strychnine Picrolonate.

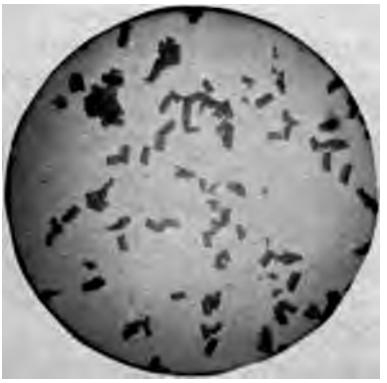


Fig. 5. Strychnine Picrate.

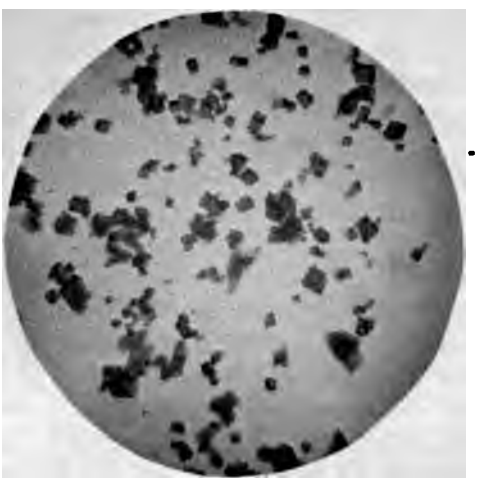


Fig. 6. Brucine Picrolonate.

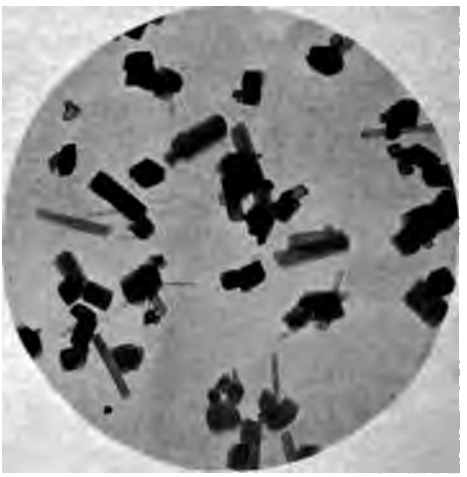


Fig. 7. Brucine Picrate.

Fig. 8. Morphine Picrolonate.

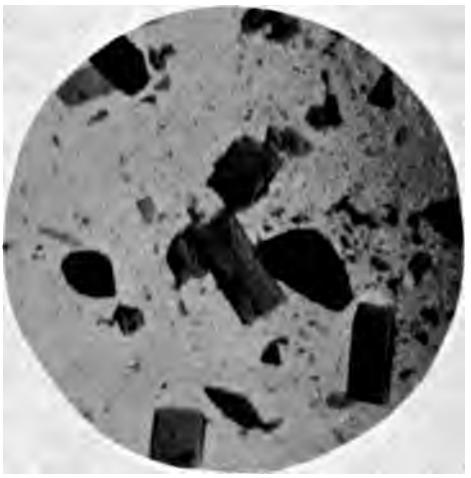


Fig. 9. Codeine Picrolonate.

Fig. 10. Atropine Picrolonate.

Fig. 11. Quinine Picrolonate.

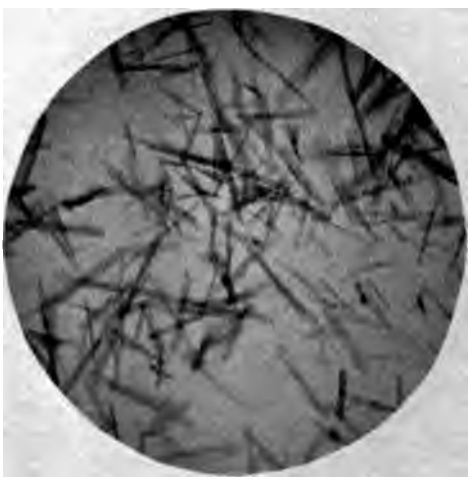


Fig. 12. Hydrastine Picrolonate.

FURTHER OBSERVATIONS ON PROTAGON.

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(Received for publication, July 13, 1907.)

The last issue of the *Bio-Chemical Journal*[1] contains a very interesting paper, by Lochhead and Cramer,[2] "on the phosphorus percentage of various samples of protagon." Lochhead and Cramer stated in an introductory paragraph that "In this communication we [they] intend to bring forward some positive evidence in favor of the view that protagon is an individual substance and not a mixture." The "positive evidence" that Lochhead and Cramer brought forward in fulfilment of that intention consists simply of less than a dozen figures for the percentage amounts of phosphorus in a few dissimilar so-called protagon[3] products obtained from ox brains with the aid of several new methods.[4] Lochhead and Cramer considered that

[1] Issued June 20, 1907 (Nos. 7 and 8).

[2] Lochhead and Cramer: *Bio-Chem. Journ.*, ii, p. 350, 1907.

[3] I say "so-called protagon products" because the paper fails to show that they were identical with the preparations universally regarded as typical, *e. g.*, Liebreich's.

[4] That these methods were valid substitutes for the processes by which the classical protagon products were made does not appear to have been determined by Lochhead and Cramer. That their methods were satisfactory substitutes, the authors seem to infer merely from the fact that most of their products contained proportions of phosphorus equal approximately to from 1.0 to 1.1 per cent. They appear to assume that this is the proportionate content of phosphorus in pure protagon. Do Lochhead and Cramer wish us to understand that the name protagon may now be applied to any white brain material having a proportionate content of phosphorus equal to from 1.0 to 1.1 per cent and do they consider that any method by which such material can be obtained is a satisfactory process for the preparation of protagon?

their figures were in "close agreement," but the analytic data for percentage phosphorus contents, of the preparations of their "most highly purified products" especially, *i. e.*, A, B, C and D (which had been recrystallized only *three* times, however) show clearly that there were, for at least three of the four *purest* products, very significant *disagreements* on recrystallization, as may be seen in the appended summary of their results (p. 354):

PERCENTAGE OF PHOSPHORUS IN PROTAGON PRODUCTS MADE BY LOCHHEAD AND CRAMER, WITH THE AID OF NEW METHODS.

Solvent Used for Extraction	Ethylalcohol			Methyl-alcohol	Ethylalcohol	Chloro-form
Samples of protagon	A	B	C	D	E	F
Crude crystalline product...	—	—	—	—	-	1.18
Recrystallization from	Ethyl-alcohol	Ethyl-alcohol	Boiling Glacial Acetic Acid	Methyl-alcohol	Methylalcohol	
First.....	—	—	—	—	a 1.22 b	
Second.....	1.34	1.25	(1.25)	0.94	1.14 1.05	
Third.....	1.07	1.05	0.96	0.97		

Why was recrystallization of products A and B discontinued after the *third* treatment, when the decrease of proportionate phosphorus content was so noticeable, and that too in the products recrystallized from *ethylalcohol*, the alcohol employed for purposes of preparation and purification in practically all previous protagon investigations? It is very probable that additional recrystallizations would have shown further lowering of the percentage phosphorus content. It is significant, also, that the first product (D) which was recrystallized from *methylalcohol* was at once different, in percentage phosphorus content, from products A and B. The proportionate contents of phosphorus in the latter were probably unusually high after the *first* recrystallization, for which, unfortunately, Lochhead and Cramer present no analytic data.

Lochhead and Cramer laid great stress on the constancy of the percentage content of phosphorus in Gamgee and Blankenhorn's protagons, even after repeated recrystallization (see footnote 2, p. 349). They regarded that constancy of proportionate phosphorus content as evidence of the complete removal of pseudocerebrin, by Gamgee and Blankenhorn, from their prota-

gons, and as proof of the purity of the latter. Lochhead and Cramer also mistakenly assumed that Posner and I obtained a product of constant composition after repeated recrystallization. If such constancy of proportionate phosphorus content is so significant, however, and implies so much in favor of the purity of the product, what opinion do Lochhead and Cramer entertain regarding their own results in this connection which, in all cases except one, showed a steady fall of phosphorus content after recrystallization. It seems to me that Lochhead and Cramer have indeed obtained, contrary to their own opinion, "different mixtures by extracting brain tissue by different methods."

The results of the treatment of products A and B are apparently very significant, from several standpoints. The statement made by Lochhead and Cramer regarding the origin of "Protagon C" is not very clear, but, as I understand it, "Protagon B," which contained 1.25 per cent of phosphorus after the second recrystallization from ethyl alcohol was then divided, and each of two portions of it was subjected to a third recrystallization; one portion from "boiling absolute alcohol," by which treatment phosphorus content was reduced from 1.25 to 1.05 per cent (B); the second portion (C) from "boiling glacial acetic acid," which caused even a greater decrease of phosphorus content—from 1.25 to 0.96 per cent. Does not Cramer recall that Koch[1] obtained phrenosin ("cerebrin")[2] from the material which is extracted from brain by boiling alcohol and separated when the extract is cooled to 0° C., *i. e.*, from protagon; that by recrystallization from glacial acetic acid two or three times, Koch thus succeeded in isolating pure, phosphorus-free phrenosin; in effect, prepared *phosphorus-free* protagon by the method Lochhead and Cramer used for the *purification* of protagon? The drop in percentage phosphorus content from 1.25 to 0.96 per cent that was caused by but one crystallization from glacial acetic acid, suggests that if crystallization had been repeated in that reagent several times, Lochhead and Cramer would have obtained phrenosin in as complete a state of purity and as free from associated matter containing phosphorus, as Koch did. Lochhead and

[1] Koch: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 138, 1902.
[2] Koch: *Amer. Journ. of Physiol.*, xi, p. 310, 1904.

Cramer said nothing about any of this, so that it is probable the entire matter was overlooked by them.[1]

Special allusion is made to protagons E and F on pp. 351 and 352 of this paper.

The significance of the above noted *disagreements*[2] is obvious when certain other data are taken into account. Lochhead and Cramer laid special stress, and properly so, upon Gamgee's results and methods. The following quotation from Gamgee's[3] remarks shows how little Lochhead and Cramer agree with Gamgee on the matter of phosphorus content of protagon as affected by the process of recrystallization.

"The amount of phosphorus in specimens of protagon which had been crystallized from alcohol *four* or *five* times was not smaller than that present in protagon which had only *once* been crystallized, though a thorough treatment with ether preceded each recrystallization."

Some of the duplicate results obtained by Gamgee in his phosphorus determinations were the following: (A) 1.094 and 1.107, (B) 1.032 and 1.081.[4] (See remarks on p. 344 of this paper.)

With regard to the results tabulated on p. 340 and which the reader sees, in the light of the remarks just made, are not in particularly close accord, Lochhead and Cramer remarked as follows, at the beginning of their summary of conclusions:

"The close agreement(!) between the phosphorus percentage of various samples of protagon prepared by the most diverse methods is strong evi-

---

[1] Besides indicating methods for the preparation of phrenosin ("cerebrin"), lecithin and kephalin, Koch said at the conclusion of one of the papers already referred to (*Zeitschr. f. physiol. Chem.*, xxxvi, p. 140, 1902, in which his observations on these three substances were summed up): "Das Protagon braucht weiter nicht berücksichtigt zu werden, da ich die Theile (phrenosin, lecithin, kephalin), aus denen es aufgebaut, direkt bestimme." In the paper in the *American Journal of Physiology* (xi, p. 324, 1904) Koch said a typical preparation of protagon (Noll's, *Zeitschr. f. physiol. Chem.*, xxvii, p. 370, 1899) "contained about 75 per cent of phrenosin (cerebrin); the remainder consisted of sulphur compound and either lecithin or more probably kephalin."

[2] Designated by Lochhead and Cramer as examples of "close agreement."

[3] Gamgee: *A text book of the physiological chemistry of the animal body*, i, p. 427, 1880.

[4] Gamgee and Blankenhorn: *Zeitschr. f. physiol. Chem.*, iii, p. 279, 1879.

dence in favor of the view that protagon is an individual substance of a well-defined chemical composition."

The second of the three sentences comprising their summary of conclusions is the following:

"Even more conclusive evidence (than that referred to in the preceding quotation) is afforded by the observation of Posner and Gies, that after ten times repeated crystallization the protagon crystals separating out *have the same phosphorus percentage as the substance in the mother liquor*."[1]

At another place in their paper they refer to this particular matter somewhat more accurately as follows:

"Posner and Gies have recrystallized protagon as often as ten times. They found the phosphorus percentage of the crystals separating out (0.93 per cent.) and of the substance in the mother liquor (1.02 per cent) to be *almost identical*[1]—surely the most conclusive proof (!) of the homogeneous nature of the substance they were dealing with. *The significance of this result does not seem to have been recognized by the authors.*[1] According to them even this sample contained considerable quantities of pseudocerebrin as evidenced by the effect of their method of fractionation."

"The significance of this result," I am happy to state, *was* duly "recognized" by us[2] when we came to the only unbiased conclusion the results could possibly justify, viz., that "the crystals separating out (0.93 per cent P)" and "the substance in the mother liquor (1.02 per cent P)" *were not identical*—that, even after a *tenth* recrystallization under the conditions maintained by us, protagon lost to the mother liquor, material which was chemically unlike that crystallized from it. This conclusion was enforced not only by a comparison of the above quoted figures for phosphorus contents, but also by the further observation that the products were unlike in appearance and consistence.

The suggestion that "the significance of this result does not seem to have been recognized" by Posner and me—a result which Lochhead and Cramer think deserves more emphasis than any of their own data, because "even more conclusive" than any of theirs in showing that protagon is *not* a mixture—leads me to believe that the following remarks, made by us[3] in this connection, were overlooked by Lochhead and Cramer:

[1] Italics are mine.
[2] Posner and Gies: This *Journal*, i, p. 108, 1905.
[3] Posner and Gies: *loc. cit.*, p. 107.

"The analyzed protagon samples (of the last product) were purified and dried as usual. Only necessary quantities were removed for this purpose. So much depended on the exactness of the results for phosphorus content of the protagon, especially of the final product, that our colleague Mr. W. N. Berg, was invited to check the results. Mr. Berg was told nothing beforehand about the characters of the products he was asked to analyze. His results agreed perfectly with the writer's. The results recorded above for proportions of phosphorus in the protagons are those obtained by Mr. Berg. We are indebted to him for his cordial coöperation."

My own result for the proportion of phosphorus in the final protagon product was 0.92 per cent; for that in the substance of the filtrate it was 1.04 per cent. These results were as closely in agreement with Dr. Berg's as *duplicate* results obtained by either of us, or any one else, doubtless would have been. Instead of giving my own results only, or the averages of Dr. Berg's results and mine, we presented only Dr. Berg's, for the reason that the difference between his two figures in this particular connection was somewhat less than between mine, and therefore slightly less favorable to the view to which our previous results consistently led us.

The agreement between Dr. Berg's independent results and ours emphasizes strongly the fact that the difference between the quoted 0.93 per cent for the *crystallized* protagon, and the 1.02 per cent for the mass of solid substance in the *mother liquor*, was a *real* difference and not merely the appearance of one created by defects of technic that would make such a mathematical difference of no chemical significance.[1] This was so obvious, we thought, that we did not dwell upon the fact, nor did we imagine it would be necessary to do so. The facts alluded to below show further how much Lochhead and Cramer ignored in their jump to the conclusion quoted above that is so wofully unappreciative of the meaning of the results of ours to which they referred.

We found that, on subjecting the specified protagon to an *eleventh* recrystallization *after ordinary drying*[2] (by the fractionation process from alcoholic solutions that were purposely made more

[1] Lochhead and Cramer failed to remark that by recrystallization the phosphorus content of our product was reduced from 1.22 per cent to 0.93 per cent.

[2] *In vacuo* over concentrated sulfuric acid at room temperature.

concentrated than those used for the first ten recrystallizations), the percentage amount of phosphorus in the first fraction of the protagon that had thus been recrystallized an *eleventh* time, but which was filtered off at 28° C. (instead of at 0° C. as theretofore), was 0.95 per cent, whereas that of the second fraction from the resultant filtrate (filtered off at 0° C.) was 1.22 per cent, and that of the solid substance in the final filtrate from these two fractions was 1.68 per cent (see p. 346 for a summary of these and related facts). On the assumption that the solid matter in the above mentioned "final filtrate" was composed solely of unprecipitated protagon[1] (0.93 per cent P) and material containing about 1.68 per cent of phosphorus, it is obvious that, if the mixture consisted of *seven* parts of the protagon (0.93 per cent P) and *one* part (12.5 per cent) of substance having 1.68 per cent of phosphorus, the proportion of phosphorus in the *mixture* would have been 1.02 per cent, the figure obtained for it by Dr. Berg.[2] If Lochhead and Cramer attach no importance to differences between analytic figures which show such evident disparity between two products as that just pointed out, I am obliged to insist, nevertheless, that due attention be paid to the real significance of our results that they would ignore and which, seemingly, in their ardor to establish what at the outset they stated they *intended* to bring forward, they would make appear to be just the opposite of what it really was.

Lochhead and Cramer have shown singular partiality to the only pair of results of the very many in our paper that could possibly bear any semblance to evidence in favor of their view that protagon is a chemical entity.[3] They ignored dozens of more

[1] Protagon cannot be completely precipitated from its solution in 85 per cent alcohol by refrigeration at 0° C. Posner and Gies: *Loc. cit.*, p. 93.

[2] My own figures suggested such a mixture of *five* parts of protagon and *one* of the same material with specially high phosphorus content.

[3] After the *ninth* recrystallization of the same protagon, in a reduced volume of solvent, the percentage of phosphorus in the filtrate was 0.96 per cent. No determination of phosphorus was made in the corresponding product that had been obtained in crystalline form. The physical qualities of the two products were different enough, however, to make it evident that if the phosphorus contents were nearly the same the mixtures were quite distinct. The phosphorus content in the filtrate material after the *sixth* recrystallization was 1.42 per cent; that of the protagon, even after the first recrystallization, was not above 1.22 per cent.

significant results of our fractionation experiments, the methods of which were, in effect, *merely fractional recrystallizations of dry protagon products by classical methods used for their preparation.* The protagon that had been recrystallized a *tenth* time and *dried*, whose phosphorus content was 0.93 per cent, and which may certainly be regarded as having been as pure as any protagon ever prepared (if the Gamgee and Blankenhorn method is acceptable for the purpose, and no one denies that), yielded the following results on simple fractional recrystallization from 85 per cent alcohol, in solutions somewhat more concentrated than those employed for the first ten recrystallizations. (Posner and Gies: *Loc. cit.*, p. 109, table xiv.)

FRACTIONATION PRODUCTS OBTAINED AT VARIOUS TEMPERATURES BY POSNER AND GIES FROM PROTAGON PREPARED BY THE GAMGEE AND BLANKENHORN PROCESS AND RECRYSTALLIZED *ten* TIMES.

Fractional Product.	Weight in Grams.			Percentage of Phosphorus.	
	Separate.		Combined.		
	28° C.	0° C.		28° C.	0° C.
I. Protagon fractions:					
A—1. Product at 28° C....	2.05			0.95	
2. " " 0° C....		1.69			1.22
B—3. " " 28° C....	0.97			0.89	
4. " " 0° C....		1.12			1.12
C—5. " " 28° C....	0.49			0.74	
6. " " 0° C....		0.59	6.91*		0.98
II. Insoluble portion (residue)...			5.39	0.74	
III. Solid matter in the corresponding filtrates from the protagon products at 0° C:					
A.....		0.60			1.68
B.....		0.31		}	1.31†
C.....		0.28	1.19		
IV. Combined ether-washings of I and II.....			2.23	0.47	
Substance recovered.....			15.72		
Substance taken.....			16.20	0.93	

* The total weights of protagon products at the two temperatures were: 28° C.—3.51 grams; 0° C.—3.40 grams.

† The residues B and C were combined for analysis because of the small quantity of each.

A sample of protagon prepared by Cramer's coagulation method, which, according to Cramer, yields typical protagon, was subjected by us to the same simple fractional recrystallization

process that was used for purification purposes, with the following results (p. 96, table viii):

FRACTIONAL PRODUCTS OBTAINED FROM CRAMER'S PROTAGON (SHEEP BRAINS) BY THE METHOD OF LESEM AND GIES.

Fractional Product.	Weight in Grams		Percentage Elementary Composition.		
	Separate	Combined.	Phosphorus.	Nitrogen.	Sulfur.
I. Protagon fractions:		13.5			
1. First product	5.9		1.29	2.08	0.68
2. Second product	3.2		1.02		0.60
3. Third product	2.3		1.00	1.90	
4. Fourth product	2.1		0.68		0.54
II. Insoluble protagon (final residue)	3.2	3.2	0.39	1.96	0.22
III. Solids in the filtrates from, and the washings of, the protagon products:		3.15			
1. First product	1.31		1.92		
2. Second product	0.79		1.48		
3. Third product	0.56		1.28		
4. Fourth product	0.49		1.02		
Substance recovered	19.85				
Original protagon	20.00		1.07	2.07	0.62

Until our methods of fractional recrystallization are shown to be inadequate, the foregoing results cannot be superseded by data that are less significant. Why is it that those who have so confidently supported the idea that protagon is a definite chemical substance never deliberately subjected their products to mechanical fractionation and the resultant fractions to comparative analysis? What more elementary or decisive test could be applied to protagon products to prove the uniformity of their characters? What is the matter with the fractionation process that it should be so studiously shunned by all the "protagonists?"

I desire to make this communication as brief as possible and will single out for immediate comment only those remaining points in Lochhead and Cramer's paper which cannot satisfactorily be reserved for a future discussion in connection with the

results of work related to protagon that has been in further progress in this laboratory.

The third of the three sentences comprising the summary of conclusions at the end of the paper by Lochhead and Cramer reads as follows:

"The view that protagon is a mixture of substances differing in their solubility and in their phosphorus contents is not compatible with these results and cannot be accepted until the substances constituting the mixture have been isolated."

One such substance, *i. e.*, phrenosin (pseudocerebrin, cerebron, cerebrin) was isolated by Gamgee, Wörner and Thierfelder, Koch, Lesem and Gies, and Posner and Gies. Koch has isolated others. Thudichum claimed to have identified phrenosin and others in protagon. See p. 354.

Lochhead and Cramer presented data showing that Gamgee's pseudocerebrin (1880) and Wörner's cerebron (1900) were practically identical. Why did they not go further and give facts showing that both these products were practically the same as Thudichum's phrenosin (1874[1]), that was first described before Gamgee discovered the difficulty of separating "pseudocerebrin" from protagon and which separation does not appear to have been made when his classical products were prepared.[2]

Lochhead and Cramer stated that "according to Gamgee, pseudocerebrin exists together with protagon and is extracted together with protagon from the brain. Being less soluble in alcohol than protagon, Gamgee was able to separate it from protagon by simple recrystallization. If his view is correct it is possible to remove pseudocerebrin entirely from the phosphorized matter, which can be obtained as a definite chemical compound of a constant phosphorus percentage." Did Gamgee ever prepare a "pseudocerebrin" product that was free from phosphorus? Did Gamgee ever prepare protagon that was free from "pseudocerebrin?" If so, where may his data on these points be found? In this connection I invite from Lochhead and Cramer answers to the following, which was published by us two years ago in a discussion of Gamgee's method of preparing pseudocerebrin, of the

[1] Gies: This *Journal*, ii, p. 159, 1906.

[2] Which on repeated recrystallization retained the same proportion of phosphorus.

significance of his product, etc., but of which no correction has yet been made that we know of (Posner and Gies: *Loc. cit.*, p. 69):

"Gamgee has apparently made no further contribution to the subject. It is difficult to understand exactly, from the very brief statement of Gamgee's that is quoted above, what is meant by the direction to subject '*impure*' protagon to '*repeated*' crystallization, for it was shown by Gamgee and Blankenhorn that the composition of ox-brain protagon, which had been recrystallized only *twice* from 85 per cent. alcohol,[1] was unchanged by a *third* recrystallization, and their results indicated in a general way that protagon from dog-brains that was recrystallized only *once* was as 'pure' as protagon from horse-brains that was recrystallized *four* times.[2] Gamgee and Blankenhorn did not take the subsequently-stated precaution to recrystallize 'repeatedly,' unless only one to 'four or five times' at most is considered sufficient to meet the requirements of the direction given. Is it not highly probable, therefore, quite certain in fact, that their protagons contained pseudocerebrin? Are not the remarks of Gamgee in 1880 indicative of a logical departure from the position held in 1879, and of a feeling that protagon, as previously prepared and described, contained the new substance subsequently discovered by him, *i. e.*, pseudocerebrin?

"How can anyone know definitely when pseudocerebrin has been completely removed from protagon in the process of recrystallizing from 80 per cent alcohol at 45° C.? Gamgee has said nothing that will answer the question. He referred, it is true, to the nodular masses in which pseudocerebrin crystallizes, but 'pure' protagon, that has been recrystallized *ten* times, forms such masses if the solution from which it crystallizes is not too dilute and the crystallization is not too slow. Is it not almost certain, from the various facts recited above, that every protagon that was described before Cramer drew attention to pseudocerebrin, contained the latter substance? Who of the 'protagonists,' except Gamgee, knew about pseudocerebrin, and who succeeded in removing it from his protagons either by design or accident? If these questions be answered in the negative, and we think they must be, what has protagon been if not always a mechanical mixture of substances?

"Was Cramer's protagon assuredly free from pseudocerebrin? If pseudocerebrin was not contained in it, how did Cramer remove it? His methods of preparation and purification warrant the conclusion, however,

---

[1] The different concentrations of alcohol (80 per cent and 85 per cent) could not account for the discrepancies noted.

[2] "The amount of phosphorus in specimens of protagon which had been crystallized from alcohol *four* or *five* times was not smaller than that present in protagon which had only *once* been crystallized, though a thorough treatment with ether preceded each recrystallization." (Gamgee: *Text-Book*, p. 427.)

that pseudocerebrin was present in each of his protagon preparations. We have separated a pseudocerebrin-like substance from every sample of protagon made by us by the Cramer method. We believe that the higher temperature prescribed in Cramer's method tends to favor the presence of even a larger proportion of pseudocerebrin in the protagon obtained by means of the new process than that in the protagon prepared by the older and more cautious methods.

"What is the effect of the 'repeated recrystallization' (necessary, according to Gamgee, to remove pseudocerebrin from protagon), on the protagon itself, or, more correctly, on the phosphorized matter associated with the pseudocerebrin? What is the composition of such associated matter, or of 'pure' protagon, if we may call it such? Gamgee gave no information on these points. No one else, we believe, is able to supply a definite answer.

"That Gamgee's further studies, unfinished though they were (1880), modified greatly his original views was shown also by his remarks on the great stability of protagon in boiling alcohol[1] and ether, and on the presence in the brain of a *free*, preformed 'non-phosphorized cerebrin.' His earlier, opposite views have been quoted repeatedly by successive investigators, all of whom, except Thudichum, have remained unacquainted, until recently, with the remarks in the text-book. It is to be regretted that Gamgee's ultimate conclusions on the matters referred to above were not published where they would have been sure to receive the attention they merited. If they had been promptly published in the journal, for example, in which the paper by Gamgee and Blankenhorn appeared, we believe the protagon question would have been settled before Parcus and succeeding authors could have made the obvious mistakes that have been recorded in abundance since."[2]

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[1] On p. 426 of his *Text-Book* Gamgee stated: "At higher temperatures than 55° C. alcohol appears to decompose protagon" On p. 429 the following may be found: "Pure protagon is remarkably rebellious to the action of even boiling alcohol, though that action be continued for hours." P. 440 records the following: "By *boiling* with alcohol *for many hours* protagon appears to be decomposed." In his first protagon paper he wrote: "Es verdient ferner hier noch erwähnt zu werden, dass wir bei der Darstellung sowohl, als auch beim Umkrystallisiren von Protagon die Lösungen nie über 45° C. erwärmten, da wir überzeugt sind, dass Liebreich mit Recht annimmt, dass sich dasselbe in alkoholischer Lösung über 50° C. erhitzt, zu zersetzen beginnt" (Gamgee and Blankenhorn: *Zeitschrift für physiologische Chemie*, iii, p. 280, 1879).

[2] The paper by Gamgee and Blankenhorn was published in 1879 (*Zeitschr. f. physiol. Chem.*, iii, p. 260). It is stated at the head of the paper that it was received for publication *May 10*. Nothing was said in that paper about pseudocerebrin, of which, a year later, however, Gamgee stated (*Text Book*, i, p. 441, 1880) "a very considerable quantity was

The following, which is quoted from Lochhead and Cramer's paper (p. 353), was evidently written to show that the method of preparing the protagon referred to yielded typical protagon and that fractional recrystallization had no effect on the composition of the product:

"Protagon E.—A sample of protagon was prepared in the manner described for samples A and B. After one recrystallization it contained 1.22 per cent P. It was recrystallized once more out of methylalcohol in the following manner; a small quantity of the solvent, which was not sufficient to dissolve all the protagon, was added first and decanted after having kept the solution boiling for one minute. The same process was repeated with another small quantity of methylalcohol. The crystals separating out in these two fractions gave on analysis:

First fraction ..... 1.14 per cent P.
Second " ..... 1.05 per cent P."

In short, a substance having a composition of 1.22 per cent of P, on recrystallization yielded two crystalline fractions, one of which contained 1.14 per cent of P and the other, 1.05 per cent of P. How

always extracted with protagon and other phosphorized matters from brain by alcohol at 45° C." When did Gamgee first learn about pseudocerebrin? Until recently the only statement by Gamgee about it appeared in his text book and few, as Thierfelder lately made evident, knew anything about its presence there (Gies: This *Journal*, ii, p. 167, 1906). Three years ago Thierfelder (*Zeitschr. f. physiol. Chem.*, xliii, p. 22, 1904) published the substance of a personal communication to him by Gamgee to the effect that his (Thierfelder's) then recently described cerebron was merely rediscovered pseudocerebrin; that the composition of the former as given in Gamgee's text book was the same as that of the latter published by Wörner and Thierfelder (*Zeitschr. f. physiol. Chem.*, xxx, p. 542, 1900); and, furthermore, that Gamgee by reference to his laboratory journal, after the appearance of Wörner and Thierfelder's paper, found, under date of *July 11, 1879*, the note that pseudocerebrin melted at 210° C. (The melting point of cerebron was found by Wörner and Thierfelder to be 209–212° C.) Lochhead and Cramer have repeated the substance of this in the form of a letter from Gamgee.

These facts explain, it seems to me, why Gamgee and Blankenhorn did not mention pseudocerebrin in their paper. Pseudocerebrin was discovered by Gamgee *after* the Gamgee and Blankenhorn products had been isolated and described, and when it was too late to effect removal of pseudocerebrin from them. And with pseudocerebrin present, Gamgee and Blankenhorn failed to affect the percentage content of phosphorus in their protagon products by recrystallization (!). In the preliminary work begun as early as *1877*, Gamgee, "assisted by Mr. Leopold Larmuth

much material remained in the filtrates and what was the phosphorus content of each mass? Did any of the original protagon remain undissolved in this treatment? If so, what was its content of phosphorus? The foregoing data appear to be the only ones bearing on fractional reprecipitation that were obtained by Lochhead and Cramer. What better evidence of the heterogeneity of the protagon product under consideration could be required to prove it a mixture? The results in this particular connection are in complete accord with those for protagons (made by the Gamgee and Blankenhorn process and by Cramer's coagulation method) obtained in this laboratory every time the fractional recrystallization process was applied to them.

A further quotation from the Lochhead-Cramer paper is appended (p. 354; also, p. 355):

"Protagon F.—The dry powder was extracted with 500 cc. of boiling *chloroform*. After cooling, 1500 cc. of *ether* were added. A white precipitate formed, which was analyzed without further purification, as only

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. . . . . found that the amount of phosphorus in specimens of protagon which had been crystallized from alcohol four or five times, was not smaller than that present in protagon which had only once been crystallized, though a thorough treatment with ether preceded each recrystallization." (*Text book*, i, p. 427, 1880.) Of course pseudocerebrin was present in these products also.

Why is it that Gamgee never candidly revised the statements in his paper with Blankenhorn that his discovery of "pseudocerebrin" made necessary in the interest of the truth about protagon? Even his text book treatment of the subject is vague and contradictory in places. How can Lochhead and Cramer expect to use the data pertaining to pseudocerebrin as a prop for their hypothesis that the protagons of Liebreich and of Gamgee and Blankenhorn (the classical products) were something uniform chemically, without focusing attention upon the real facts in the case; without emphasizing the apparent truth of the situation to which Thudichum referred when he declared that Gamgee's change of position in 1880 from that assumed in 1879 was an outcome of Thudichum's fractionation studies of protagon in 1879 and that "Gamgee himself, by using my [Thudichum's] method for the preparation of phrenosin, extracted this principle from protagon and to hide its identity called it pseudocerebrin?" (Thudichum: *The progress of medical chemistry*, p. 191, 1896.) I do not know that these statements by Thudichum have ever been refuted. I have already endeavored to show the fact that phrenosin, pseudocerebrin and cerebron were essentially the same substance. (Gies: This *Journal*, ii, p. 159, 1906.)

small quantities could be obtained in this way. It contained 1.18 per cent P[1]. . . . . Sample F, prepared by precipitation with ether, is of special interest, as, according to Gies, protagon contains a considerable quantity of an ether soluble substance, poor in phosphorus. If this is correct, the method employed for Sample F should yield a substance richer in phosphorus than the other samples of protagon. This was not found to be the case."

Sample F was found to contain 1.18 per cent of P, which *is* relatively high for protagon, if the tabulated data presented by Lochhead and Cramer from the papers of the leading investigators is to be the basis for judgment. But how do Lochhead and Cramer know that "Sample F" was protagon? Lochhead and Cramer speak of this matter, however, so far as it pertains to our observations, as if their own results were the only evidence besides ours that could be brought forward, apparently being quite oblivious of the fact that Kossel and Freytag obtained from an ether extract, a protagon having a rather high content of phosphorus, *i. e.*, 1.35 per cent. Furthermore, the above quotation disregards the fact that our remarks were especially directed to "*freshly precipitated* (hydrated)" protagon, a distinction which was not noticed by Lochhead and Cramer. We stated (p. 111) that "long continued extraction of pure *dry* protagon with ether in a Soxhlet apparatus failed to affect its composition—another matter totally ignored by Lochhead and Cramer. Besides, we said nothing at all about protagon solubilities in *ether-chloroform* solutions. The solvent action of ether on one hand and of *ether-chloroform* on the other may be very different, both in kind and in degree.

Lochhead and Cramer present a summary of figures apparently intended to show an impressive agreement between their lowest data and those obtained by previous workers, for the proportion of phosphorus in protagon. With the exception of the figure for Liebreich's protagon, all the others given by them range between 1.13 per cent (Ruppel) and 0.93 per cent (Posner and Gies). The quoted figure for Liebreich's is 1.23 per cent, *i. e.*, an average of his two results (1.1 and 1.1 per cent) for products from

[1] Why was it called protagon? Because it was white, had been obtained from brain and contained 1.18 per cent of phosphorus?

domestic animals and the one (*1.5 per cent*)[1] for protagon from human brain. The quoted figure for Kossel and Freytag's products is 0.97 per cent, no account being taken of their protagon from an ether extract having 1.35 per cent. Relatively high results obtained by Lesem and Gies, *e.g.*, 1.23 per cent, for products very carefully prepared by the Gamgee and Blankenhorn method and particularly low figures for similar products, *e. g.*, 0.57 per cent, were not referred to by Lochhead and Cramer. Zuelzer's[2] low figure (0.72 per cent) as well as the low one obtained by Gamgee and Blankenhorn (0.72 per cent), after treatment with hot ether, is also not mentioned, and Ulpiani and Lelli's[3] high result (1.31 per cent) is ignored. The figure quoted by Lochhead and Cramer for Posner and Gies' products is 0.93 per cent, which fails to indicate the much higher and also the decidedly lower results obtained by us for many typical protagon products. Thudichum's important data were entirely ignored. It seems to me the reader is never particularly interested in only one side of such questions, but invariably appreciates a presentation of both. When all the figures for phosphorus content of protagon products that have been obtained by the classical methods are assembled, they at once suggest the question, What *is* the percentage amount of phosphorus in protagon? Who can answer this question?

Lochhead and Cramer say that "in order to definitely deprive a substance like protagon, *possessing characteristic physical and chemical properties*,[4] of its existence as a chemical individual,[5] it is necessary to isolate and identify the various substances constituting the mixture—a task which, in view of the differences in the physical and chemical properties of these substances should be a comparatively easy one." Would not the "isolation and

[1] Did Liebreich have two different protagons or was the high figure (1.5 per cent) expressive chiefly of analytic error? In either event what does the amount, 1.23 per cent, as given by Lochhead and Cramer, stand for?

[2] Zuelzer: *Zeitschr. f. physiol. Chem.*, xxvii, p. 255, 1899.

[3] Ulpiani and Lelli: *Gazetta chimica italiana*, xxxii, p. 466, 1902.

[4] Italics are mine.

[5] Who, since Thudichum first successfully pointed out its heterogeneity, has established the properties of protagon? Has protagon been deprived of anything that ever justly belonged to it?

identification" of *only one* of them settle the point? Phrenosin (pseudocerebrin, cerebron, cerebrin) has already been isolated from protagon and identified, has it not? As to the "comparative ease" of the task alluded to, we fear Lochhead and Cramer have passed very lightly over facts too well known to require discussion.[1]

Lochhead and Cramer suggested that "a final and decisive proof of the homogeneous nature of a substance even of a less complex nature than protagon, cannot be brought about until the substance in question can be synthesized. In default of this," they added "*the constancy of its physical and chemical properties*[2] is generally considered to be sufficiently trustworthy evidence."

In view of the great *uncertainty* that has prevailed for many years regarding the "*physical and chemical properties*" of protagon, it would surely help greatly to a speedy and correct solution of the protagon problem if Lochhead and Cramer were to publish a detailed statement of the physical and chemical properties which they appear to think are so constant and which, by implication, must be so well known—the properties, for example, that were so definite for and characteristic of their "Sample F," let us say, to which I referred on p. 352. Has the "homoprotagon" accessory to the main hypothesis been abandoned? If not, what are the properties of homoprotagons that distinguish them from *the* protagon? Cramer did not return to this subject in his last paper.

The work on protagon that Cramer has described thus far has not, it seems to me, been directed at the heart of the question, regarding protagon, that he appears to be desirous of solving. That question is not: Can products closely resembling the pro-

[1] "There can be no doubt that protagon is accompanied in the brain by large quantities of a body or bodies which may provisionally be conveniently classed under the term of cerebrin, and likewise by smaller quantities of other phosphorized bodies, containing a percentage of phosphorus very close to that found in lecithin, yielding the same products of decomposition, and *the separation of which presents extraordinary difficulties.*" (Gamgee: *Text Book of Physiological Chemistry*, i, p. 429, 1880). See also Halliburton: The Oliver-Sharpey Lectures, 1907; *British Medical Journal*, May 4 and 11, 1907.

[2] Italics are mine.

tagon of Liebreich, and of Gamgee and Blankenhorn, be prepared by *new* methods, but rather, are the protagon products prepared by the Liebreich and the Gamgee and Blankenhorn methods or any other, samples of a *definite chemical individual?* The problem is not, it seems to me, merely the production of *something new to look like the old* and to which the familiar name may be applied but the establishment, clearly and precisely, of the qualities of the old classical protagon products of debatable characters; *if* they are in fact samples of a *substance* and not of *mixtures*.

The remarks in the appended quotation, published by us two years ago, are quite as applicable now as we thought them then:[1]

"We have already indicated the probability that all preparations of protagon that were made previous to Cramer's work contained phrenosin. We obtained phrenosin-like products from every sample of protagon made by us by Cramer's method, that we examined. The fact expressed in the last sentence of the quotation (below)[2] from Cramer's remarks on the results obtained by Lesem and Gies must also be explained away satisfactorily before it will be possible for any one to insist that protagon prepared by the classical methods in general use before the appearance of Cramer's paper was not always a mixture. Cramer prepared protagon by a new method but did not investigate the question whether protagon was a mixture. He did not subject his new protagon to a fractiona-

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[1] Posner and Gies: *Loc. cit.*, p. 76.

[2] "By treating protagon, the analysis of which gave figures corresponding to other authors', with dilute alcohol for several times, Lesem and Gies got substances which differed widely in their percentage of phosphorus and in their solubility in alcohol. . . . . The decreasing percentage of phosphorus in the 'precipitated protagon' corresponds entirely with the statement of Gamgee regarding pseudocerebrin. We may therefore assume that the 'protagon' of Lesem and Gies is a mixture of protagon and pseudocerebrin. Indeed, the precipitate of the first extract, where the protagon should be purest, has a percentage of phosphorus equal to that of protagon. It remains to explain how it is that this mixture of a phosphorus-free with a phosphorus-holding substance should on analysis give a percentage of phosphorus corresponding with that of protagon, when one would, of course, expect a lower figure. This deficit must have been made up by a substance richer in phosphorus than protagon itself. This substance is indeed present in Lesem and Gies' protagon, for in the filtrate from the freshly precipitated protagon of the first extract and in the alcohol-ether washings we find a substance containing a higher percentage of phosphorus."

tion process and consequently left untouched the fundamental point alluded to in the last sentence in the remarks of his we have quoted.[1]

"Suppose it be admitted that Lesem and Gies, and all other protagon investigators to the time of Cramer's work, analyzed products containing phrenosin (pseudocerebrin, cerebron), and we think as indicated before that this must be conceded, would it not also be admitted at the same time that the idea contended for so long unavailingly by Thudichum, and recently by Lesem and Gies, that protagon was a mixture, was correct? But what was the nature of the phrenosin-free part of the mixture that has always been called protagon? If now we decide to apply, as Cramer does after Gamgee, the name protagon to the phrenosin-free part of the old protagon, do we not merely shift our point of view and give to a part the name that has heretofore been applied to the whole?

"Did any one ever succeed in separating and identifying this hypothetical protagon—the part of protagon that would remain if phrenosin were removed from it? If we admit Cramer's conclusion that Lesem and Gies were dealing with mixtures of such *hypothetical* protagons and phrenosin, then surely we are also obliged to admit that it was this newly conceived protagon in their mixtures that yielded the products of relatively high and distinctly *variable* phosphorus content that were alluded to in the quotation above.[2] But if Cramer's view were correct would not this hypothetical protagon also have to be conceived as a mixture? It stands to reason also that after removal of the phrenosin from the protagon mixture, the phosphorus content of the remainder would be much higher than any ever before recognized as typical of protagons."

I have lately completed, with Mr. Matthew Steel, an inquiry into the general nature of Ulpiani and Lelli's "paranucleoprotagon." Our results are about to be published. The non-protein part of paranucleoprotagon behaves much like ordinary protagon when subjected to mechanical fractionation, and it seems to be a mixture of products similar to those in protagon.

I hope to publish in the near future, also, the results of a study of the effects of desiccation on the fractional recrystallization of

[1] "Dr. Cramer informed us that the experimental part of his work was completed before the publication of the paper by Lesem and Gies, and that it was only lack of proper opportunity to take up the work again that prevented him from going into this matter."

[The only results of such fractional recrystallization that Cramer has published since the above was first written, two years ago, that I know of, are referred to on p. 351 of this paper.]

[2] "The products of high phosphorus content could not have been impurities, because the methods of Lesem and Gies, and their analytic results, furnish conclusive evidence against that possibility."

protagon. That the solubility of protagon in 85 per cent alcohol at 45° C., and in ether, was decreased by drying was made very clear in the work described in the paper I published with Posner. That preliminary conversion of protagon to the anhydrous condition favored the fractionation results obtained by us was quite obvious.

IS THE CONDUCTION OF THE NERVE IMPULSE A CHEMICAL OR A PHYSICAL PROCESS?

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I.

The remarkable resistance of nerves to fatigue and the difficulty of demonstrating satisfactorily the existence of chemical changes as the result of stimulation of the nerve are too well known to require restatement or discussion. The experiments of Bowditch, Wedenski and others, in which nerves were stimulated for many hours without indications of fatigue, are described in the common text-books. The failure to detect the liberation of heat from a stimulated nerve is equally well known. From these experiments the conclusion has very frequently been drawn that the transmission of the nerve impulse is a purely physical, molecular, and not a chemical process. In connection with these facts and the much investigated electro-motive phenomena of nerve a mountain of physical hypotheses as to the nature of the nerve impulse has been built up.

On the other hand it has often been argued that all other "life processes" with which we are familiar are associated with some sort of chemical change and that it would not be safe to decide upon purely negative evidence that the propagation of the nerve impulse is a unique exception. It is possible to conceive that fatigue does occur, but that recuperation takes place so rapidly that the recovery can be completed in the short intervals between the successive shocks of the Faradic current. The attempts to secure positive evidence of chemical change in stimulated nerve have taken in the main four forms:

1. *The attempt to prove fatigue of the nerve.*

Passing by the earlier experiments, the results of which were wholly negative or the methods admittedly faulty, I merely

mention some of the more recent attempts. Gotch and Burch[1] found that when two stimuli separated by a very brief interval of time were applied to a frog's sciatic, no electro-motive response to the second shock was obtained if the interval was less than about 0.006 of a second. They also noted that the duration of this "critical interval" was very greatly influenced by temperature.

According to Garten[2] definite fatigue could be produced in the olfactory nerve of the Pike by the use of induction shocks occurring as frequently as every 0.27 of a second. In this case the evidence of fatigue was the failure of the electro-motive response.

2. *The attempt to demonstrate the presence of the products of chemical change in stimulated nerve.*

Again I pass by the older observations. Waller[3] has shown that tetanization of a nerve for a few minutes increases its subsequent electro-motive response to induction shocks, and that a similar increased response can also be obtained by exposing the nerve for a short time to very dilute carbon dioxide. From these experiments he argues the probability of the production of carbon dioxide in the stimulated nerve. The observation is extremely interesting, but the argument that the cause of the increased electro-motive effect is the same in both cases is certainly not convincing.

Bethe[4] has shown that certain substances, fibril acids, in the neurofibrillæ, have their distribution altered by the passage of a constant current through the nerve, in such way that the readily staining fibril acids migrate away from the anodal region and collect at the cathodal. He assumes provisionally that the conditions of anelectrotonus and catelectrotonus are connected with this movement, and that changes in the fibril acids, or in their relation to the neuro-fibrillæ, have to do with the propagation of the nerve impulse. In order, however, to secure the characteristic difference between anode and cathode, as brought out by staining, the current must be allowed to act for an exceedingly

[1] Gotch and Burch: *Journ. of Physiol.*, xxiv, p. 410, 1889.

[2] Garten, quoted by Biedermann in *Ergeb. d. Physiol.*, ii, 2, p. 129.

[3] Waller: *Lectures on Physiology*, First Series, London, 1897.

[4] Bethe: *Allgemeine Anatomie und Physiologie des Nervensystems*, Leipzig, 1903.

long time compared to that in which stimulation and conduction can occur. It would not be safe to assume that these changes are the essential factors in nerve excitation and conduction.

3. *The attempt to demonstrate a change of temperature in stimulated nerve.*

Helmholtz[1] working with a thermopile could find no evidence of evolution of heat in the frog's sciatic when stimulated.

Heidenhain[2] obtained similar results.

Schiff[3] believed that he could show with the thermopile the production of heat in stimulated nerves of the cat and rabbit.

Rolleston,[4] using an exceedingly sensitive electrical resistance thermometer, could find no change of temperature in nerve as the result of stimulation.

4. *The attempt to prove the necessity of free oxygen to the nerve.*

According to Baeyer[5] a nerve surrounded by an atmosphere of pure nitrogen or hydrogen loses its irritability in about five hours, and becomes again excitable within a few minutes after the readmission of oxygen. He compared the effect of lack of oxygen upon stimulated and unstimulated nerves and could find no well marked difference between them. So far as one may draw conclusions from his experiments they seem to indicate that stimulation and conduction in the nerve are not oxidation processes.

II.

Neither the experiments mentioned above nor other work along similar lines have for so far given conclusive evidence as to the chemical or non-chemical nature of the nerve impulse. In the effect of temperature on reaction velocity van't Hoff[6] and Arrhenius[7] have pointed out a simple and reliable criterion for the discrimination between chemical processes and those which are usually designated purely physical processes. They have shown that a rise of 10° of temperature increases the velocity of a chemical

[1] Helmholtz: *Arch. f. Anat. u. Physiol.*, p. 158, 1848.
[2] Heidenhain: *Studien d. physiol. Inst. zu Breslau*, iv, p. 250, 1868.
[3] Schiff: *Arch. de physiol. norm. et pathol.*, p. 157, 1869.
[4] Rolleston: *Journ. of Physiol.*, ii, p. 208, 1890.
[5] Baeyer: *Zeitschr. f. allgem. Physiol.*, ii, p. 169, 1903.
[6] Van't Hoff: *Études de dynamique chimique*, Amsterdam, 1884.
[7] Arrhenius: *Zeitschr. f. physikal. Chem.*, iv, p. 226, 1899.

reaction to two or three times its original rate, while no known physical process is accelerated in anything like so great an amount by a similar rise of temperature, probably not more than 5 to 15 per cent at the most. This principle has been used by Loeb[1] to show that the artificial maturation of the eggs of *Lottia* is really a chemical process. Mr. C. D. Snyder was also asked by Professor Loeb to investigate the effect of temperature on the reaction velocity of the tortoise heart. These experiments gave a high temperature coefficient and indicated definitely that chemical processes underlie the rhythmic activity of the heart.[2] His observations were fully confirmed by Robertson using the heart of a crustacean, *Ceriodaphnia*.[3] The same criterion has been used by Loeb[4] to determine whether the production of artificial parthenogenesis by the action of hypertonic sea water depends upon changes of a chemical or of a physical nature. The high coefficient obtained showed conclusively that the process is a chemical one. Professor Loeb also asked Dr. Burnett and myself to investigate the effect of temperature on the velocity of the nerve impulse, since it would be possible by this means definitely to decide whether the propagation is due to chemical or to non-chemical processes. Dr. Burnett found it advantageous in the beginning of his experiments to study the effect of temperature on the latent period of striated muscle. This part was published early in 1906.[5] Afterward on account of the pressure of other work Dr. Burnett was unable to continue the investigation, and, at Professor Loeb's request, I have carried it through with the results herein reported.

The fact that the velocity of the nerve impulse is affected by temperature was observed by Helmholtz[6] and has been frequently noted since, but so far as I know, no investigations have heretofore been made with the direct purpose of determining the temperature coefficient of nerve conduction. Nicolai[7] made a

[1] Loeb: *University of California Publications*, Physiology, iii, p. 1, 1905.
[2] Snyder: *Ibid.*, ii, p. 125, 1905.
[3] Robertson: *Biol. Bull.*, x, p. 242.
[4] Loeb: *University of California Publications*, Physiology, iii, p. 40, 1906.
[5] Burnett: This *Journal*, ii, p. 195, 1906.
[6] Helmholtz: *Arch. f. Anat. u. Physiol.*, p. 358, 1850.
[7] Nicolai: *Arch. f. d. ges. Physiol.*, lxxxv, p. 65, 1901; *Arch. f. Physiol.*, 1905, supplement, p. 341.

series of experiments on the speed of the nerve impulse in the olfactory of the Pike, in which, among other things, he considered the effect of temperature. He used the "negative variation" photographically recorded as the indicator of the passage of the impulse. His results show indeed that the velocity is affected by the temperature, but the experiments were not specifically planned for the determination of the temperature coefficient and for several reasons are inapplicable to that purpose. The length of nerve employed was relatively small, from 4 to 23 millimeters, allowing room for large experimental errors. The temperature changes were, except in one experiment, all made in one direction; that is, the temperature was either raised for each successive observation, or lowered for each, so that it was not possible to know whether the observed change in rate was all due to the change in temperature, or whether it was also affected by other variables. The importance of this consideration I shall discuss more fully in connection with my own experiments. Von Miram[1] studied the effect of high temperatures on the conductivity of the frog's sciatic. His results are given in the form of curves and averages. On account of the great individual variations a statistical method is absolutely out of the question in dealing with the effect of temperature off nerve conduction and hence von Miram's paper is unavailable for the purpose. His table shows, for example, at 15° a minimum rate of 25 meters and a maximum rate of 38.7 meters a second, and at 30° a minimum of 38.7 and a maximum of 49.5 meters. Since, according to this, one nerve at 15° may have a higher rate than another at 25° it is evident that one cannot safely draw conclusions from averages except from an impossibly large number of observations. Besides, the high rate of the impulse in the frog's nerves makes it necessary to deal with differences of thousandths of a second—differences in many cases smaller than the probable experimental error.

Snyder[2] has based on Nicolai's and v. Miram's experiments calculations of the temperature coefficient of the velocity of the nerve impulse, and concludes that "a comparison of tempera-

[1] Von Miram: *Arch. f. Physiol.*, p. 533, 1906.
[2] Snyder: *Arch. f. Physiol.*, p. 113, 1907.

ture coefficients leaves us no longer in doubt as to the nature of nerve conduction." The methods employed in calculating these coefficients are not free from objection. He says, "I have used only the average values of the determinations of these authors." At first glance it is hard to see how these averages were made. Of the eight lines in the table founded on Nicolai's tables,[1] I quote as specimens the first two and the last.

t_1	t_2	R_1	R_2	$\frac{10 R_1}{R_2 (t_1 - t_2)}$
9.25	3.45	13.7	5.65	4.1
9.25	4.82	13.7	8.55	3.6
25.0	3.45	22.2	5.65	1.8

In the above table at rate 13.7 temperature 9.25 is obtained by averaging two consecutive observations in the experiment of November 11 in which the stimulus was the closing of a constant current. The single items are

9° rate 14.6
9.5° " 12.8

Compared with this is rate 5.65 at temperature 3.45 obtained by combining and averaging as follows:

3.7°	rate	6.4	from above mentioned experiment of November 11.
3.5°	"	6.4	
3.4°	"	5.7	from an experiment of November 30, using break induction shock as stimulus.
3.2°	"	4.1	
Average 3.45°	"	5.65	

In the second line the same 13.7 rate is used as before. The rate 8.55 at temperature 4.82 is secured by the following selection:

5.0°	rate	7.6	from above experiment of November 11.
4.7°	"	7.1	
4.8°	"	10.2	from an experiment of November 28, break induction shock.
4.8°	"	9.3	
Average 4.82°	"	8.55	

[1] *Arch. f. d. ges. Physiol.*, lxxxv, pp. 82 and 83.

Finally the rate of 22.2 at temperature 25° was the last response of the dying nerve used in the experiment of November 11, and the only observation recorded at that temperature. It will thus be seen that the tables in Snyder's paper are obtained by selecting and comparing a few observations made upon the nerves of different animals and in different degrees of freshness. One of these nerves, moreover, is expressly stated by Nicolai to have been injured by lying on ice. By a different selection of data a different conclusion might be reached.

III.

Our own experiments herein described were all made upon the pedal nerves of the giant slug, *Ariolimax columbianus*. The importance of this selection is seen in the great length of nerve available and the slowness of the impulse, Jenkins and Carlson[1] having found a mean rate of 440 millimeters a second, while it is possible to use a nerve 100 millimeters or more in length. If, on the other hand, the frog's sciatic were employed one would expect to deal with magnitudes of the following order: Suppose a distance of 30 millimeters between the near and far points of stimulation and at a certain temperature a rate of 30 meters a second. This would mean a difference in length of latent periods for the near and the far points of 0.001 of a second. Now suppose at another temperature the rate is 45 meters a second; the difference of the latent periods becomes one one-thousand-five-hundredth of a second. The value of the desired quotient will thus depend upon a time relation of the difference between one one-thousandth and one one-thousand-five-hundredth, or one three-thousandth of a second; or, if one uses to measure the time a tuning fork of 200 double vibrations a second, it depends on the measurement of one-fifteenth of a single wave. It is at once apparent that the probable error in such a calculation is vastly larger than in observations on the pedal nerves of *Ariolimax* in which one may obtain differences between the latent periods for the near and the far points of from four to thirty-hundredths of a second.

[1] Jenkins and Carlson: *Amer. Journ. of Physiol.*, viii, p. 251, 1903.

The methods at first employed were essentially those of Jenkins and Carlson[1] except that the moist chamber in which the animal was placed was so arranged that its temperature could be regulated by surrounding it with warm or cool water as desired. It was soon found that this plan was impracticable because when the muscle is cooled the latent period is enormously lengthened, the muscle contracts very slowly, and the total shortening is greatly reduced. It thus becomes difficult at low temperatures to secure curves with sharply defined beginnings. When the higher temperatures were used another difficulty was found in the increasing tendency to spontaneous rhythmical contractions. For these reasons it was necessary to leave the muscle outside the moist chamber and to cool the nerve only.

After many trials the method finally adopted was as follows: a moist chamber was constructed of heavy copper foil, 80 × 25 × 25 millimeters in size. This was soldered by one end to the inner wall of a metal case 120 × 105 × 40 millimeters inside measurement. The outer case was provided with an inlet tube, through which water could be circulated, and a stirrer to equalize the temperature of the water. In the attached end of the moist chamber and the wall of the outer case was cut a V-shaped slit about 10 millimeters deep. The edges of this slit and the end of the case in its neighborhood were coated with wax to prevent any possible contact of the nerve or muscle with the metal. The use of copper foil for the moist chamber made it necessary very carefully to avoid touching it with the nerve, which rested on the electrodes only. It was found that a moist chamber of hard rubber did not respond with sufficient promptness to changes of temperature for the purposes of this investigation.

The electrodes consisted of platinum wires sealed into slender glass tubes to insure perfect insulation. These tubes were then fastened in place by means of sealing wax. One pair of electrodes was placed near the V-shaped slit about halfway from bottom to cover of the moist chamber, the other pair was near the opposite end of the moist chamber and at a similar depth. The pairs of electrodes in part of the experiments were 32 millimeters, in the others 64 millimeters apart. They were connected

[1] Jenkins and Carlson: *Amer. Journ. of Physiol.*, viii, p. 251, 1903.

with the poles of an inductorium by means of a Pohl's commutator without cross wires, so that the stimulating current had the same direction in both pairs of electrodes.

The stimulus employed consisted of the break induction shock from a Zimmermann's DuBois-Reymond inductorium of 10,000 windings, with one Edison-Lalande cell, Type Z, in the primary circuit. The circuit was broken automatically by a peg projecting from the rim of the kymograph drum. The time was recorded by a tuning fork making 100 double vibrations a second.

Two large percolators connected by a Y-tube to the inlet tube of the outer case served as reservoirs of warm and cold water for the regulation of the temperature. A thermometer held by a rubber support was so placed that its bulb was near the middle of the moist chamber at the level of the nerve.

The muscle used consisted of about two or three centimeters of the posterior end of the foot. This was pinned firmly at its anterior end to a block which carried a small pulley. A hook was placed in the free, posterior end of the preparation, and a thread passed from the hook around the pulley, and up to a light lever arranged to record the contractions on the drum of the kymograph.

The muscle of the slug is so readily thrown into strong and lasting tonic contraction that special care had to be taken to get it into proper position before beginning the dissection. It was also found much better to secure the slug in its elongated position than to attempt after dissection to stretch the nerve to the length which it would have in the relaxed animal. In doing the latter one frequently finds that the adhering blood and mucus are much stronger than the nerve itself and, stretching irregularly, they tend to break or injure the nerve. For these reasons in making the preparations a second block was placed in front of the one bearing the pulley. The animal was placed on the blocks with the posterior end directed toward the pulley and allowed to creep until only about two centimeters of its length remained on the block bearing the pulley. Pins were now driven firmly through the body into this block very near its edge. The pinning usually acted as a stimulus to cause the animal to creep straight forward. When maximal elongation had been obtained in this way the anterior end was suddenly pinned down firmly, the body was

slit open and the viscera removed. A thread was tied around the pedal nerves near the pedal ganglion (the two nerves taken together made the best preparation), and their side branches were cut off as far back as the first pins. The nerve was then held up by the thread, care being taken to have it fully extended, and the body was cut through at the line of contact of the two blocks. The block with the preparation was now placed in front of the V-shaped slit in the moist chamber. A pledget of cotton wet with the animal's blood was laid in the bottom of the slit, the nerve was extended across the two pairs of electrodes and held in position by looping the thread around a pin fastened at the far end of the moist chamber. The upper side of the slit was closed by another loose pledget of cotton wet with blood. The nerve touched only this moist cotton and the two pairs of electrodes. The moist chamber, which had been lined with thoroughly wet filter paper, was now closed with a glass plate covered with wet filter paper.

The velocity of the nerve impulse was determined by the method of Helmholtz. In its application, however, to the nerve of the slug certain difficulties must be mentioned. In order that the curves obtained by stimulation at the near and the far points respectively shall rise with equal steepness from the horizontal and so avoid error in determining the exact beginning of each curve, it is usual to employ a maximal stimulus. With the slug muscle, subject as it is to enormous changes of tonus, it is impossible to say what constitutes a maximal stimulus. The only practical thing to do is to use a strength of shock which experience proves to give fairly equal curves. If the curves do not come out well the tracing must simply be thrown away. Much time and many tracings were lost on account of this difficulty. I found it best to use fairly strong induction shocks, the secondary coil at from 275 to 300. If, however, the current employed was too strong, while the curves obtained looked very satisfactory, it often happened, that when the tuning fork record was counted the latent periods for the far and the near points were practically equal. What occurred was probably this, that on account of the high potential of the induced current and the poor electric conductivity of the nerve the effect was essentially the same as in the so-called unipolar stimulation;

the whole length of the nerve was excited simultaneously whether the near or the far electrodes were used. How easily the limit of allowable strength may be overpassed is shown by one experiment in which with the coil at 260 the latent periods for the two points of stimulation were equal while with the coil at 275 the results were apparently rational.

The nerve-muscle preparation of the slug is astonishingly sensitive to mechanical stimulation. I found, for example, that when the tuning fork was set in vibration before starting the kymograph the slight jar communicated to the table excited a contraction. For this reason it became necessary to support the tuning fork in a manner entirely independent of the table on which the moist chamber rested. For the same reason it was necessary to discard the ordinary brake used to start and stop the Ludwig kymograph and to employ a device which would work more smoothly.

The tendency to spontaneous rhythmical contraction frequently interfered with the experiments. The rate of these contractions was always comparatively slow, usually not far from one a minute, and it was mostly possible to secure curves in the quiet intervals of relaxation which gave apparently rational results. Still it was impossible to be sure that the state of excitability of such a muscle would not so vary from moment to moment as to affect the length of the latent period and thus render unreliable the estimation of the rate of the impulse. On this account most preparations showing a persistent tendency to spontaneous contractions were thrown away as useless.

The contraction and relaxation of the slug muscle are relatively slow, especially the latter, and at times the muscle when once stimulated tends to remain for a considerable period in a state of strong tonic contraction. When this occurs it, of course, interferes seriously with accurate velocity determinations. I found, for example, that if the second curve was taken not more than half a minute after the first the results appeared usually to be reasonably consistent, but if a period of one or two minutes intervened absurd or poorly agreeing figures were obtained. It would seem that changes taking place in the muscle, impossible to control, affect the duration of the latent period. This makes it necessary to avoid all unnecessary delay between the two

curves. After considerable experience with this sort of difficulty, I found it best to throw away those tracings which I had marked "relaxation slow" without taking the trouble to count the vibrations. In order to shorten the time between the curves for the near and the far points I put two pegs for the circuit breaking key on the rim of the kymograph drum 180° apart and adopted a rate of speed which allowed the essential part of the tracings to be taken within one half a revolution. This did away with the necessity of a readjustment of the drum between the two steps of the experiment. It also had the advantage of allowing twice as many tracings to be taken on the one drum.

In order to secure the quotient

$$\frac{\text{Velocity at } T + 10 \text{ degrees}}{\text{Velocity at } T \text{ degrees}}$$

in which T and $T + 10$ are absolute temperatures, it was thought preferable to make the observations at temperatures of exactly 10° difference rather than to make calculations from observations at other or random intervals. The plan usually followed was to take two observations at some convenient temperature; then by the method previously described to change the temperature 10° and take two observations, after which the nerve was brought back to the original temperature and two more determinations were made. The object in taking two determinations at each temperature was, of course, that they should serve as controls on each other. The reason for not taking more than two is that, while the nerve remains irritable for some hours after preparation, it nevertheless undergoes changes, degenerative or otherwise, which affect its conductivity. In order then that the chief variable shall be the change of temperature it is important that the observations shall follow each other as rapidly as possible. It is not possible, however, to secure a change of 10° without a very considerable interval of time. It required usually five to eight minutes to bring about the desired change and be sure that the temperature would remain fairly constant during the observation. It was of course necessary that the nerve should have time to take the actual temperature indicated by the thermometer. As an

extreme illustration of the progressive changes which may occur, apart from the change due to temperature, I quote from the tables to follow the velocities found in experiment 8:

Temperature.	Velocity in Centimeters.
21°	46 } Average 42.
21°	38 }
11°	25 } Average 23.5.
11°	22 }
21°	29 } Average 28.5.
21°	28 }

In the above it is evident that neither the quotient

$$\frac{42}{23.5} \text{ nor } \frac{28.5}{23.5}$$

correctly represents the acceleration due to a rise of 10°, but that for temperature 21° the mean of the first and last observations,

$$\frac{24 + 28.5}{2}$$

should be taken, giving a quotient of

$$\frac{35.25}{23.5}$$

In a preceding section I stated that on account of the failure to observe the order of experiment just mentioned—*i. e.*, a return to the beginning temperature—the data given by Nicolai and by v. Miram are not usable in calculating the temperature coefficient. Jenkins and Carlson[1] working with the pedal nerves of *Ariolimax* observed that "the rate of the nervous impulse seems to vary with the freshness of the preparation." Nicolai[2] called attention to similar changes in the Pike's olfactory and

[1] Jenkins and Carlson: *Loc. cit.*, p. 254.
[2] Nicolai: *Arch. f. Physiol.*, supplement, p. 376, 1905.

considered them to be the effect of the repeated stimulation. I am strongly inclined from some observations of my own to believe that these changes occur in the absence of repeated stimulation, and that they are caused by degenerative processes in the nerve fiber when separated from its cell body, but I have not sufficiently investigated the matter. The fact that they do occur renders the above order of procedure absolutely necessary.

In the course of my experiments I found that the preparations remained irritable for many hours, but that it was not advisable to make more than a few observations on each nerve, and then only while it was in the freshest condition possible. It is obvious that the nerve in the moist chamber is exposed to the influence of many unknown or uncontrollable variables, and that the effect of these must increase as the preparation becomes older. The possible injurious effect of repeated stimulation, contact with injurious substances, changes in moisture, etc., may occur. Other, accidental, disturbances may easily arise. For example I several times found that the nerve suddenly ceased to respond to the strength of shock which had just been giving good curves, and discovered that with the lowering of the temperature the moisture condensing on the nerve and electrodes had short-circuited the current. Changes also take place in the muscle as well as the nerve. This was evidenced by the increasing tendency to spontaneous contraction. These contractions were not caused by the condition of the nerve in the moist chamber for they were not affected by cutting the nerve off close to the muscle.

In the following table of results of my experiments I have given not only the calculated velocities and the temperature coefficients obtained from them, but also the latent periods for the near and far points in each experiment, in order that the reader may have an opportunity to judge of the degree of consistency and of variability in the individual experiments. Each numbered experiment represents a fresh preparation. The figures are given in the order in which the records were made. The intervals between the time of stimulation at the near and the far electrodes were, as stated above, not far from half a minute. The intervals between any two observations at the same temperature were usually from one to three minutes; and

the intervals between observations at different temperatures were from five to eight minutes.

Column 1 is the number of the experiment. Column 2 is the temperature. Column 3 is the latent period for the far point, and Column 4 for the near point, in hundredths of a second. Column 5 is the velocity in centimeters per second. In Column 6 the average velocity for each temperature is placed in line with the figure representing that temperature in Column 2. Column 7 is the quotient

$$\frac{\text{Velocity at } T + 10}{\text{Velocity at } T}$$

in which T and $T+10$ represent the temperatures in the experiment.

1 Exp. No.	2 Temp. °C.	3 Latent Period. Far.	4 Latent Period. Near.	5 Velocity.	6 Average Velocity.	7 Velocity at $T + 10$ / Velocity at T
1	21	22	17	70		
	11	27	20	*50*		
	11	31	16	22	36	
	21	22	14	44	57	1.58
2	22.5	16.5	10	48		
	12.5	24	14	32		
	12.5	26	15	29	30.5	
	22.5	20	14	54	51	1.67
3	21	24	17	46		
	21	24	17.5	49		
	11	31.5	22	34		
	11	36	24	27	30.5	
	21	29.5	18.5	29		
	21	28	21	46	42.5	1.39
4	9	44	29	21		
	9	38	27–	29		
	19	28.5	19	34		
	19	31	16	21	27.5	
	9	38	22.5	21		
	9	39	20	12	20.75	1.32
5	22.5	22.5	15	43		
	12.5	27	16.5	30		
	12.5	31	20.5	30	30	
	22.5	26.5	24	*128*	85.5	*2.85*

6	21	18	12.5	58		
	21	17	11.5	58		
	11	25.5	15	30		
	11	26	16	32	31	
	21	20	14.5	58		
	21	20	15	64	59.5	1.92
7	21	30.5	14	53		
	21	20	13.5	49		
	11	26	15	29		
	11	27.5	17.5	32	30.5	
	21	22	14.5	43		
	21	24	14.5	34	44.75	1.46
8	21	23	16	46		
	21	24	13.5	38		
	11	34	21	25		
	11	37	22.5	22	23.5	
	21	32	21.5	29		
	21	30.5	19	28	35.25	1.50
9	21	19	12	46		
	21	21	12	36		
	11	24.5	13.5	29		
	11	28.5	13.5	21	25	
	21	22.5	13	34		
	21	21	11.5	34	37.5	1.50
10	21.5	22	16.5	58		
	21.5	21	14.5	49		
	11.5	23.5	17	49		
	11.5	26.5	18	38	43.5	
	21.5	22	15.5	49	52	1.19

1	2	3	4	5	6	7
Exp. No.	Temp. ° C.	Latent Period. Far.	Latent Period. Near.	Velocity.	Average Velocity.	Velocity at $T + 10$ / Velocity at T
11	21.5	33.5	24.5	36		
	11.5	40	24.5	21	21	
	21.5	26	19	46	41	1.95
12	20.5	20.5	13	43		
	10.5	25	16	36		
	10.5	26	16.5	34	35	
	20.5	19	12	46	44.5	1.27
13	21	23	17	53		
	21	25	15	*32*		
	11	23.5	17.5	*53*		
	11	27	18.5	38	40.5	
	21	22	15	46	43.75	*1.08*
14	21	17	11.5	58		
	21	19	11	40		
	11	20.5	15	*58*		
	11	27.5	16	28	43	
	21	21	12.5	38		
	21	19	13	53	47.25	*1.10*
15	11.5	26.5	16	30		
	11.5	27	13.5	24		
	21.5	17	14	*106*		
	21.5	19.5	13.5	53	79.5	
	11.5	25.5	16.5	36		
	11.5	28.5	18.5	32	30.5	*2.60*

16	21.5	22	14.5	43		
	11.5	28	16.5	28	28	
	21.5	24.5	17.5	46	44.5	1.59
17	21	23	17	53		
	21	21	16.5	*71*		
	11	31	18	25		
	11	34	21	25	25	
	21	26	18	40		
	21	26	19.5	53	54.5	*2.17*
18	21	19.5	12	43		
	21	22	14	40		
	11	26	16	32		
	11	27	15	27	29.5	
	21	21	14	46		
	21	19.5	13	49	44.5	1.51
19	23	25	20	64		
	23	25	20	64		
	13	30.5	22	38		
	13	34	24.5	36	37	
	23	28	22.5	58		
	23	26.5	21	58	61	1.65
20	21	27.5	21	53		
	21	28	17	29		
	11	34	23	29		
	11	33.5	22	28	28.5	
	21	25	18.5	53		
	21	22	20	*160*	73.75	*2.58*

1	2	4		5	6	7
Exp. No.	Temp. °C.	Latent Period.		Velocity.	Average Velocity.	Velocity at $T + 10$ / Velocity at T
		Far.	Near.			
21	21	25	15.5	34		
	21	26.5	16	30		
	11	35.5	20.5	21		
	11	36	21	21	21	
	21	30	21	36		
	21	31	21.5	34	33.5	1.59
22	21	27	17.5	34		
	21	27.5	19.5	40		
	11	34.5	23	28		
	11	36.5	23.5	25	26.5	
	21	29	21	40		
	21	31.5	25	49	40.75	1.54
23	23	22	16	53		
	23	23.5	18	58		
	13	28.5	20	38		
	13	33	22.5	30	34	
	23	34	20.5	*24*		
	23	36	22	*27*	40.5	*1.19*
24	21.5	25.5	17.5	40		
	21.5	26	18	40		
	11.5	30.5	19.5	29		
	11.5	33	20	25	27	
	21.5	28	19	36		
	21.5	27	18.5	38	38.5	1.43

25	25	25	20	64		
	25	25	20.5	71		
	15	31	20.5	30		
	15	31	22.5	34		
	5	38.5	28	*30*		
	5	46	29.5	19	24.5	
	15	41	25	20		
	15	39	25.5	22	26.5	*1.08*
	25	32	24	40		
	25	27	23.5	*91*	66.5	*2.51*
26	14	27	16	29		
	14	28	18	32		
	3.5	47	26	15		
	4	45	23.5	15	15	
	14	29.5	20	34	31.7	2.11
	24	25	18	46		
	23.5	24	17	46		
27	12	41.5	32	34		
	12	42	32	32		
	1	58	40	18		
	0.5	68	48	16		
	13	50	39	29		
28	13	34	25.5	38		
	13	36	26.5	34		
	3	47	31	20		
	2	55	37	18		
	3	58	40.5	18	19	
	12	47	37	32		
	13	47	33	40	37.3	1.96

1	2	3	4	5	6	7
Exp. No.	Temp. °C.	Latent Period.		Velocity.	Average Velocity.	Velocity at $T + 10$ / Velocity at T
		Far.	Near.			
29	13	29	18	29		
	13	32.5	18	24		
	3	40.5	23	18		
	3	44	22.5	15	16.5	
	13	33	11.5	28		
	13	33	12	27	27	1.64
30	14	34	18	20		
	14	38	19.5	17	18.5	1.43
	4	47	22	13		
	4	48	24	13	13	
	14	40	23	19		
	14	38.5	20.5	18		
	24	30	17	25		
	24	31	18	25	25	1.32
	14	33	17	20		
	14	37	20	19	19	
31	15	26	17	36		
	15	27.5	18.5	36		
	5	47	23	23		
	5	47	24.5	26	24.5	
	15	26.5	17.5	36		
	15	28	19.5	38	36.5	1.49
	25	26	19.5	53		
	25	28.5	19	34		

32	25	18	15	*107*		
	25	20	16	80		
	15	26.5	18	38		
	15	28	19	36		
	5	41.5	26	21		
	5	41	27	23	22	
	15	29	20	36		
	15	29	25.5	51.5	50.25	*2.28*
33	26	19	14.5	71		
	26	17	14.5	*128*		
	16	22	16	53		
	6	41.5	28	24		
	6	42	28	23	23.5	
	16	31.5	20	28		
	16	27.5	17	30	37	1.57
	26	24	15	36		
	26	26	17	36	67.25	1.81
34	13	28	19	36		
	13	28.5	19.5	36		
	3	61.5	35	12		
	3	60	39	15	13.5	
	13	43	23.5	34	35.5	2.61
35	11	23.5	16	43		
	11	26	18	40		
	1	36	21.5	22		
	1	41	25	20	21	
	11	32	22	32		
	11	31.5	20	28	35.75	1.70

1 Exp. No.	2 Temp. °C	3 Latent Period. Far.	5 Latent Period. Near.	5 Velocity.	6 Average Velocity.	7 Velocity at $T + 10$ / Velocity at T
36	10.5	29	20.5	38	35	2.06
	11	31.5	21	32		
	0.5	51	19	17	17	
	1	49	32	19	19	
	10.5	41	31	32		
	11	33	25	40	36	1.89
37	11	33.5	23	30		
	11	40	24	20		
	1	53	20	16		
	1	54	18.5	17	16.5	
	11	44	31	25		
	11	49	30	17	23	1.39
38	11	20.5	16	71		
	11	27.5	18	34		
	1	50	30	16		
	1	54	29	13	14.5	
	11	40	29	29		
	11	36	26	32	41.5	2.87
39	10	25	17	40		
	10	28	17	29		
	0	38.5	24	22		
	−1	47	26	15		
	0	45	27	18	20	
	10	43	22	15		
	10	33	21.5	28	28	1.40

40	9.5	44	25	34		
	9.5	45	28	38		
	-0.5	81	41	16		
	-0.5	95	46	13	14.5	
	9.5	67	42	26		
	9.5	61	37	26	31	2.14
41	9.5	42	32	64		
	-0.5*	81	46	18		
	-0.5*	86	47	16	17	
	9.5	57	42	43	53.3	3.15
42	10	63	39	26		
	0	98	54	14	14	
	10	66	41	26	26	1.86
43	11	36.5	18	35	31	
	10	40	19	30		
	1	69	33	18	18	1.72
	0	88	43	14	14	
	10	49	27	29	29.5	2.11
	11	49	25	27		

*In Experiment 41 the contractions were very small at 0.5°; the determinations are in consequence less reliable than in the other experiments.

In the tables above the highest quotient is 3.15 and the lowest is 1.08. The average of the whole number of quotients obtained is 1.78. Inspection of the table will show that in nearly all, though not all, cases where the quotient is very much above or very much below the general average the abnormal result is due to some one, or in a few cases to some two, figures which are notably out of accord with the other magnitudes in the experiment. These numbers are printed in italics. Thus in experiment No. 5 the high quotient, 2.85, is clearly due to the effect of the velocity 128 at 22.5° which differs so far from the other velocity at that temperature, namely, 43. Again in Experiment No. 14 the low quotient 1.10 is the effect of the apparently disproportionately high rate of 58 at 11°. The significant fact is that out of 48 quotients, obtained from 43 different nerves, only two are low enough to come within the limit of acceleration of a physical process by a temperature rise of 10°. It seems logical to conclude from the outcome of these experiments that the transmission of the nerve impulse is a chemical process.

Admitting the chemical nature of the nerve impulse, the question arises, can we go further and reach any conclusion as to the particular kind of reaction involved? It would be dangerous to carry speculation very far in this direction, but one thing appears to be significant and that is the relatively low reaction velocity as compared with that of those biological processes which are known to involve oxidations or the giving off of carbon dioxide. Among such processes may be mentioned the germination of seeds, the segmentation and development of the egg and the contraction of muscle. According to the experiments of Clausen[1] the quantity of carbon dioxide given off from germinating seeds for temperatures between 0 and 25° increases about 2.5 times for each rise of 10° temperature. Loeb[2] has found that free oxygen is necessary to the production of artificial parthenogenesis, and that a rise of temperature of 10° hastens the process three or more times. For the rate of contractions of the terrapin heart Snyder[3] obtained at temperatures between 0 and

[1] Cohen: *Lectures on Physical Chemistry*, New York, p. 60, 1902.

[2] Loeb: *University of California Publications*, Physiology, iii, p. 39, 1906.

[3] Snyder: *University of California Publications*, Physiology, ii, p. 141, 1905.

25° quotients ranging from 10.2 to 2.1 For the sinus venosus of the frog's heart he found quotients of similar magnitudes,[1] as well as for the rhythmic contraction of smooth muscle.[2] It will be noticed further that in the experiments on contractions of the heart and of smooth muscle the quotient for temperatures below 15° become markedly larger. The quotients for velocity of the nerve are not only lower throughout the range of temperature which I have investigated, but they do not show the excessive increase below 15°. The average of the whole 48 quotients is, as I have stated, 1.78; the average of the 19 cases in which the upper temperature was not above 15° is 1.94—an increase but not such a marked increase as is shown by contractile tissue. The inference from these facts would seem to be that the reaction involved in the nerve impulse is not of the nature of an oxidation, an inference which is made more probable by the fact that the nerve can remain irritable for several hours in lack of oxygen,[3] whether the nerve is stimulated during that time or allowed to remain at rest. One remembers also in this connection the remarkable sparseness of blood supply to the nerve trunks.

The results of this series of experiments may be summarized in a single statement: The temperature coefficient of the velocity of the nerve impulse indicates definitely that the conduction is a chemical process, but probably not of the nature of an oxidation.

[1] Snyder, *Arch. f. Physiol.*, p. 118, 1907.
[2] *Ibid.*, p. 126.
[3] Baeyer: *Zeitschr. f. allgem. Physiol.*, ii, p. 169, 1903.

QUANTITATIVE METHODS WITH HEMOLYTIC SERUM.[1]

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In a previous paper,[2] it was pointed out that the application of *direct* quantitative methods is at times impossible with hemolytic serum; that, for example, the direct determination of the amount of hemolytic amboceptor (specific thermostable substance) remaining in a heated[3] hemolytic goat serum, after the serum has been exposed to washed sheep corpuscles, is out of the question. Work has been undertaken, during the last two years, to devise a possible *indirect* method of analysis, applicable to this problem.

Such amboceptor determinations are impossible, because heated hemolytic goat serum is so changed by contact with sheep corpuscles, that duplicate titrations do not agree. To illustrate, in one attempt, duplicate titrations gave results varying from 32 per cent to 77 per cent of the original amboceptor; in another attempt, from 70 per cent to 113 per cent, depending in each case on the volume analyzed.[4]

Heated hemolytic serum contains, in addition to amboceptor, a large number of unknown substances. These for convenience have been spoken of collectively as constituting the *third serum component.*[5]

It was found that a change in the relative amount of amboceptor and third component in a heated hemolytic serum, is sufficient to render the direct analysis of that serum impossible, by causing a non-agreement of duplicate titrations. Duplicate determinations with such altered sera gave results varying from 52.5 per cent to 64.0 per cent in one experiment, and from 5.0

[1] Work aided by the Rockefeller Institute for Medical Research.

[2] This *Journal*, i, pp. 213–218, 1906.

[3] 55°–59° C., for 30–60 minutes.

[4] *Journ. of Infect. Dis.*, ii, p. 490, 1905.

[5] *Ibid.*, iii, pp. 647–662, 1906.

per cent to 9.8 per cent in another, depending on the volume analyzed; the actual amount of amboceptor present in the two experiments being 50 per cent and 5.6 per cent.[1]

These disagreements, though sufficient to render quantitative work valueless, are not as marked as the disagreements observed in sera after exposure to corpuscles. Changes, other than those of the relative amount of amboceptor and third component, were, therefore, suspected.

It was found that pure third component (heated normal goat serum) suffers marked qualitative changes when exposed to sheep corpuscles. A third component originally hemolysis-increasing (auxilytic) has its auxilytic power decreased by such exposure, or even replaced by an antilytic power; while a third component originally antilytic has its antilytic power increased.[2]

It was further found that washed sheep corpuscles give off a powerful antilytic substance into physiological saline, and that the addition of a proper amount of this exposed salt solution to a third component, produces approximately (though not exactly) the same changes as those resulting from exposure to corpuscles.

The amount of the exposed salt solution necessary to produce this approximate change, however, is not the same with all sera. With one third component, the desired change is most closely simulated, by the addition of an equal volume of the salt solution; with a second serum, a half-volume produced the closest approximation; while, with a third sample, a double volume is required.

As a result of this work, the following changes are now conceived to take place in a heated hemolytic goat serum during exposure to washed sheep corpuscles: (*i*) A decrease in the amount of amboceptor, due to the absorption of amboceptor by corpuscles. This, of course, is as yet a purely hypothetical change, as an unchallengeable measurement of this decrease is at present impossible. (*ii*) A resulting change in the relative amount of amboceptor and third component. (*iii*) The giving off into the serum of antilytic corpuscles products, the amount (and possibly the nature) of which is influenced by the nature of the serum

[1] *Journ. of Infect. Dis.*, iii, p. 648, 1906.

[2] A detailed account of these experiments is now in press in the *Journal of Infectious Diseases*.

used. And (*iv*) certain minor changes, the nature of which is not understood.

These conceived changes are so complex, and each has such a marked influence on hemolytic power, that *it does not seem possible, at present, to devise an indirect method of analysis by means of which the residual amboceptor in an exposed heated hemolytic serum can be measured.*

A NEW STANDARD FOR USE IN THE COLORIMETRIC DETERMINATION OF IODINE.

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(Received for publication, July 19, 1907.)

Of the methods which have been proposed and used for the determination of small amounts of iodine in such substances as thyroid glands, blood, muscle, and various animal and plant substances the one which has apparently been found the most satisfactory is that adapted by Baumann and Roos[1] from the old process of Rabourdin and at present known as Baumann's method. According to this process the weighed sample of material which should contain not more than a few milligrams of iodine is moistened with water and mixed thoroughly with about five times its weight of powdered sodium hydroxide. After warming gently the heating is increased until the mass fuses to a liquid or semi-solid paste. If much charred organic matter is present a little sodium nitrate may be added for its more complete oxidation, and the heating continued until a clear melt is obtained. After cooling, water is added and the aqueous solution containing all of the iodine in the form of an inorganic salt is filtered into a separatory funnel. The solution is acidified with sulphuric acid while being kept cool and is then treated with 10–20 drops of 1 per cent aqueous sodium nitrite solution and the liberated iodine shaken out with chloroform. The intensity of the pink color in the chloroform layer is proportional to the amount of iodine present, and it is only necessary to compare this color with standards of known iodine content in order to ascertain the amount of iodine in the unknown sample. The standards so far used have been prepared from aliquot portions of standard potassium iodide solution, shaking out the liberated iodine with the same volume of chloroform used for the unknown sample.

[1] *Zeitschr. f. physiol. Chem.*, xxi, p. 489, 1895–96.

The experience of Baumann and of many other investigators, has shown that the pink colors in the chloroform layers fade rapidly and therefore it is absolutely necessary that the standards be freshly prepared for practically all determinations. Many efforts have been made in this laboratory to prevent or even retard the fading of the iodine color in the chloroform but without success. In endeavoring to overcome this difficulty in another way, an examination of the tints of the aqueous solutions of a large number of pink dyes was made, in the hope of finding one, which resembled near enough the color of the chloroform solution of iodine, to be used as a standard for the colorimetric estimation of the latter. Although none of the several dozen pink dyes which were examined gave solutions possessing a tint matching exactly that of iodine dissolved in chloroform, several gave colors which corresponded near enough for all practical purposes. Among these may be mentioned Neutral Red, Rubin (patent acid) and Fuchsin S (acid fuchsin according to Weigert), all of which dyes in the present case bore the labels of Dr. G. Grübler & Co., Leipzig. The Fuchsin S was, on the whole, found to be the most satisfactory and was selected for use in the tests here mentioned.

Experiments in making the iodine determinations with standards of iodine in chloroform indicated that a very satisfactory color was one having the intensity produced by 1.0 milligram of iodine dissolved in 10 cc. of chloroform. To reproduce such a tint with Fuchsin S it was found necessary to dissolve 0.02 gram of the dye in 200 cc. of water made acid with 3–5 per cent of hydrochloric acid and dilute in turn 10 cc. of this solution to 200 cc. with water similarly acidified with hydrochloric acid. A standard prepared in this way and compared in a Duboscq colorimeter against a solution of 1.52 milligrams of iodine dissolved in 10 cc. of chloroform gave a series of readings the average of which was 14.95 of the Fuchsin S solution equaled 10 of the chloroform solution, from which it is seen that $\frac{1.52}{14.92} = 0.102$ per 1 cc. or 1.02 milligram of iodine equivalent to 10 cc. of the standard Fuchsin S solution. Similar readings were made with other Fuchsin S solutions prepared fresh and allowed to stand various lengths of time and it was found that the desired tint could be

reproduced at pleasure and remained unaltered for at least three months, provided the solutions were kept acid. In addition to this standard corresponding to 1.0 milligram of iodine per 10 cc. of solution it was also found advantageous to prepare a series of test tubes with gradually increasing intensities of the pink color corresponding to 0.025, 0.05, 0.075, 0.10, 0.15, 0.20 and 0.25 milligram of iodine per 5 cc., and also in smaller test tubes a series corresponding to 0.01, 0.02, 0.03 and 0.04 milligram iodine per 1 cc. Provided with such standards it is a simple matter to shake out the liberated iodine from the solutions of the melt of the samples examined, with 1, 5 or 10 cc. of chloroform as the amount of iodine might require and compare the solution so obtained with the corresponding standards.

The several points of advantage, such as saving of chloroform, permanence and uniformity of standards resulting through the use of such pink dye solutions as above described will be readily recognized by anyone making frequent colorimetric determinations of iodine. The dye standards have been in use in this laboratory with entire satisfaction for several months.

Attention is to be directed also to the applicability of the above described method to the rapid estimation of iodine in many pharmaceutical preparations—such as U. S. P. desiccated thyroid glands, etc.

Finally, it is of interest to note that Dr. J. H. Kastle of the Hygienic Laboratory has found that the pink colors obtained with Griess's reagent for nitrites closely corresponds with the color of iodine dissolved in chloroform. He has therefore made use of an acid solution of Fuchsin S as a permanent standard for nitrites in potable waters with good results.

ANTI-INULASE.

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INTRODUCTORY.

The peculiar endoenzyme *inulase* was first described by Green[1] in *Helianthus tuberosus*, although Dragendorff[2] had earlier noticed the inulin-splitting process in several vegetable structures. Our knowledge of this enzyme in certain fungi is largely due to the investigations of Bourquelot.[3] Subsequently, in confirmation of the result of Bourquelot, Dean[4] found inulase in the fungi *Aspergillus niger* and *Penicillium glaucum*. Inulase has not yet been found in the animal body.[5]

Since the discovery of anti-emulsin,[6] anti-enzymes have been subjected to increased experimental study by several investigators.

Anti-trypsin,[7] anti-rennin,[8] anti-cynarase,[9] anti-coagulin,[10] anti-

[1] *Annals of Botany*, i, p. 223, 1888.

[2] St. Petersburg (1870), cited by Wortmann: *Biol. Centralbl.*, iii, p. 266.

[3] *Compt. rend. de l'Acad. des sci.*, cxvi, p. 1143, 1893; *Compt. rend. de la soc. biol.*, ix, p. 481 1893; *ibid.*, ix, p. 653, 1893; *Bull. soc. mycol., France*, ix, p. 230, 1893; *ibid.*, x, p. 235. Also cf. Chevastelon, *Journ. Pharm.*, iv, p. 1, 1895.

[4] *Botan. Gazette*, xxxv, p. 24, 1903.

[5] Chittenden: *Amer. Journ. of Physiol.*, ii, p. xvii, 1898; Richaud: *Compt. rend. de la soc. biol.*, iii, p. 416, 1900; Bieri and Portier: *ibid.*, p. 423.

[6] Hildebrand: *Arch. f. pathol. Anat. u. Physiol.*, cxxxi, p. 5, 1893.

[7] Fermi and Pernossi: *Zeitschr. f. Hyg.*, xviii, p. 83, 1894; Landsteiner: *Centralbl. f. Bakteriol.*, xxvii, p. 357, 1900; Achalme: *Ann. de l'Inst. Pasteur*, xxv, p. 737, 1901; also von Dungern: *Münchner med. Wochenschr.*, 1898, cited by Metchnikoff, 1901, and by Morgenroth, 1899.

[8] Morgenroth: *Centralbl. f. Bakteriol.*, xxvi, p. 349, 1899; *ibid.*, xxvii, p. 721, 1900. Cf. Briot: *Thèse de la faculte des sci. de Paris*, No. 4, cited by Metchnikoff, 1901; also Rödén, Ref. in Maly's *Jahresber. f. Thierchem.*, xvii, p. 160, 1887.

[9] Morgenroth, cited by Nuttall: *Blood Immunity and Blood Relationship*, p. 16, 1904.

[10] Bordet and Gengou: *Ann. de l'Inst. Pasteur*, xv, p. 129, 1901.

urease,[1] anti-pepsin,[2] and also anti-anti-rennin[3] were early considered.

The presence of many of the antibodies, as well as of the common enzymes also, normally in serum, complicates the study of the development and specific relationship of the anti-enzymes and the respective enzymes. The absence from the organism of a substance like inulase and of its antibody also, then, presents opportunities especially favorable for the study of the relationship of enzyme to antibody in general and in particular of inulase and anti-inulase. I have accordingly thought it of interest to study the antiserum prepared by immunization with suspensions of ground up aspergillus. By the comparative quantitative inhibitory effects of the serum on the action of aspergillus extracts in inulin and in sucrose digestions, it seemed possible to individualize an inulase and a sucrase rather than to ascribe the two effects of the aspergillus extracts to a single enzyme. Further, by the specificity of the aspergillus serum for the aspergillus sucrase as contrasted with the sucrase of intestinal origin, there was offered a means of differentiating the aspergillus and intestinal sucrases.

EXPERIMENTAL.

In my experiments rabbits were employed. The serum was diluted up to three or four times its original volume with water, before using, to prevent the formation of a large coagulum by heating. The digestive mixtures, containing sodium fluoride and covered with liquid paraffine, stood for varying periods in test tubes at a fixed temperature, usually in the incubation room at 38°. Then they were filtered through small filters and 6 cc. samples were pipetted into large test tubes after dilution with water to a definite volume or without dilution when there was only a small quantity of sugar present. The test tubes were placed together in a net basket. Fifteen cc. portions of Fehling's

[1] Moll: *Beitr. z. chem. Physiol. u. Path.*, ii, p. 344, 1902 (reviewed by Jacoby, *Centralbl. f. Bakteriol.*, xxxii, p. 439).

[2] Sachs: *Fortschritte der Medizin*, cited by Michaelis and Oppenheimer, p. 399, 1902. Cf. Pugliese and Coggi, Maly's *Jahresber. f. Thierchem.*, xxvii, p. 832, 1897.

[3] Korschun: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 141, 1902.

solution (previously diluted one-half with water) were heated in a water-bath and rapidly transferred at the same moment into the digestion mixtures. The combined portions were then heated three minutes in a boiling water-bath; the precipitated cuprous oxide was filtered off at once upon a Gooch crucible, and weighed as cupric oxide.

The inulin employed was obtained from Merck. The white preparation was further purified by repeated washing with water and then with alcohol. The inulase was from *Aspergillus niger* and was prepared by Dr. A. L. Dean, who kindly furnished me with some of his product for the present work. The mould had been grown on an inulin-containing medium until the spores were well developed. The mycelia were filtered out of the culture fluid, disintegrated, washed in acetone, and after drying below 50° were pulverized. The product acted strongly on inulin and saccharose, but not on starch.

I. *Is there an Inulin-splitting Enzyme or an Anti-inulase present in Normal Rabbit Serum?*

In all, nine observations on the serum (in part heat-inactivated) of six normal rabbits were made. Some of the typical protocols are given. Three additional experiments to determine the influence of serum on the hydrolysis by weak hydrochloric acid are of interest in reference to my results with the inulase. An example of these, also, is included below.

The experiments show a partial inhibition of the action of the inulase when the rabbit serum is present in the digestive mixture. The same effect, however, was obtained with the heated serum and in experiments with neutralized proteid-free filtrates of the coagulated serum.

EXPERIMENT III, RABBIT 3.

	CuO in 6 cc. gm.
2 per cent inulin solution, 3.5 cc. + 5 per cent NaF solution, 1 cc.	
+ Normal serum, 1.5 ⟶ 5.0 cc.* + water, 0.5 cc.	0.0028
+ " " 1.5 ⟶ 5.0 " (boiled) + water, 0.5 cc.	0.0034
+ " " 1.5 ⟶ 5.0 " + 1 per cent inulase solution, 0.5 cc.	0.0084
+ " " 1.5 ⟶ 5.0 " (boiled) + 1 per cent inulase solution, 0.5 cc.	0.0085
+ Water 5.0 cc. + 1 per cent inulase solution, 0.5 cc.	0.0139
+ " 5.5 "	none

The digestive mixtures were kept at 38° for 20 hours. Rabbit 3 was subsequently used for immunization.

* In all my experiments, "1.5 ⟶ 5.0 cc." indicates that 1.5 cc. of the original serum was made up to 5.0 cc. by the addition of 3.5 cc. of water.

EXPERIMENT VI, RABBIT 5.

	CuO in 6 cc. gm.
1 per cent inulin solution, 6.5 cc. + 5 per cent NaF solution, 1 cc.	
+ Normal serum, 0.5 ⟶ 1.5 cc. + 1 per cent inulase solution, 1 cc.	0.0087
+ " " 0.5 ⟶ 1.5 " (boiled) + 1 per cent inulase solution, 1 cc.	0.0082
+ " " 0.5 ⟶ 1.5 " + 1 per cent inulase solution (boiled), 1 cc.	0.0017
+ " " 0.5 ⟶ 1.5 " (boiled) + 1 per cent inulase solution (boiled), 1 cc.	0.0021
+ Water, 1.5 cc. + 1 per cent inulase solution, 1 cc.	0.0151

The digestions were kept at 38° for 20 hours.

EXPERIMENT VIII, RABBIT 5.

2 per cent inulin solution, 5 cc. + 5 per cent NaF solution, 1 cc.	
+ Normal serum, 1 ⟶ 3 cc. (at 65° for 30 min.) + 1 per cent inulase solution, 1 cc.	0.0097
+ " " 1 ⟶ 3 " (boiled) + 1 per cent inulase solution, 1 cc.	0.0102
+ " " 1 ⟶ 3 " + 1 per cent inulase solution, 1 cc.	0.0111
+ " " 1 ⟶ 3 " + water, 1 cc.	0.0016

The digestions were kept at 38° for 20 hours.

EXPERIMENT IX, RABBIT 6.

1 per cent inulin solution, 6.5 cc. + 5 per cent NaF solution, 1.0 cc.	
+ Normal serum, 0.5 ⟶ 1.5 cc + 1 per cent inulase solution, 1 cc.	0.0085
+ " " 0.5 ⟶ 1.5 " (boiled) + 1 per cent inulase solution, 1 cc.	0.0078
+ Proteid-free* { I 1.5 " + 1 " " " " 1 "	0.0079
+ Proteid-free* { II 1.5 " + 1 " " " " 1 "	0.0081
+ Water 1.5 cc. + 1 per cent inulase solution, 1 cc.	0.0129

The digestive mixture was kept at 38° for 16 hours.

* Three cc. of serum were diluted up to 9 cc. in a test tube, coagulated by boiling and the addition of a trace of dilute acetic acid; the filtrate was neutralized with dilute soda solution.

EXPERIMENT XI, RABBIT 7.

	CuO in 2 cc. gm.
0.1 per cent HCl solution,* 5.0 cc. + 2 per cent inulin solution, 3.5 cc.	
+ Normal serum, 0.5 ⟶ 1.5 cc	0.0142
+ " " 0.5 ⟶ 1.5 " (boiled)	0.0146
+ Water 1.5 cc.	0.0148

The digestive mixtures were kept at room temperature for 20 hours.

* The digestions contained, therefore, 0.05 per cent HCl.

The results of my experiments with normal rabbit serum show no evidence of the presence of an inulin-splitting enzyme. On the contrary, the addition of the serum to the digestions retards the hydrolysis considerably. This noteworthy inhibition of the action of inulase by the normal serum is not due to the proteids present or to the reaction of the serum; for when the proteids are coagulated out, the neutralized filtrates still inhibit the splitting of the inulin. The retardation cannot be ascribed to the presence of an anti-inulase of the common anti-enzyme type in the serum.

Without considering for the present the nature of the products formed, it is evident that the process of hydrolysis by acids and that by the enzyme are different, since the acid hydrolysis is not influenced by the serum.

II. The Effects of the Serum of Immunized Rabbits upon the Digestion of Inulin by Inulase.

Suspensions of the aspergillus inulase preparation were ground up with physiological salt solution and kept overnight in the ice-box. Injections of the material thus freshly prepared were made subcutaneously on alternate days into two rabbits (rabbits 2 and 3, weighing 1995 gms. each) which had been bled from the ear veins for normal serum previous to the initial treatment. These rabbits were given a total of 0.42 gram each of the aspergillus powder in gradually increasing doses of from .005–0.1 gram over a period of 26 days. Rabbit 2 had lost about 200 grams in weight and Rabbit 3 about 95 grams, when the two were again bled five days after the final injection.

The digestion experiments were carried out in the same general way as were the observations on the normal serum. In the four experiments made with the serum of the immunized rabbits, it is shown conclusively that the anti-aspergillus serum inhibits the action of the inulase on inulin. An anti-inulase has been developed by repeated injections of the aspergillus. A typical protocol follows:

EXPERIMENT XIII, ANTI-ASPERGILLUS SERUM FROM RABBIT 2.

		CuO in 6 cc. gm.
	1 per cent inulin solution, 6.5 cc. + 5 per cent NaF solution, 1 cc.	
+ Serum	0.3 ⟶ 1.5 cc. + 1 per cent inulase solution, 1 cc.	0.0045
+ "	0.3 ⟶ 1.5 " (boiled) + 1 per cent inulase solution, 1 cc.	0.0132
+ "	0.3 ⟶ 1.5 " " + 1 " " " " 1 " (boiled)	0.0027

Digestions were kept at 38° for 2 days.

III. *The Effects of the Anti-aspergillus Serum upon the Inversion of Sucrose.*

The well known inverting action of yeast on cane sugar was attributed to an enzyme by Berthelot,[1] who gave to it the name "ferment glucosique." The sucrases of fungi have been described in numerous papers[2] and the animal sucrases have been studied by many investigators since the classic discovery of

[1] *Compt. rend. de l'Acad. des. sci.*, ii, p. 980, 1860. Cf. Dubrunfant: *ibid.*, xxiii, p. 38, 1846.

[2] Cf. Béchamps: *ibid.*, xlvi, p. 44, 1858; Gayon: *ibid.*, lxxxvi, p. 52, 1878; Kossmann: *Bull. soc. chim.*, xxvii, p. 251, 1877; Duclaux; *Chimie Bioliogique*, Paris, 1883, cit. J. R. Green: *The Soluble Ferments and Fermentation*, p. 115, 1899; Bourquelot: *Compt. rend. de la soc. biol.*, p. 237, 1898.

sucrase in the intestine.[1] Sucrose injected intravenously reappears in the urine unaltered, according to Cl. Bernard.[2] This indirect evidence of the absence of sucrase in the blood is in harmony with the experimental observations reported below. Mendel and Mitchell,[3] however, have recently observed in one experiment that cane sugar intraperitoneally injected may be utilized in part.

It has already been suggested that the sucrases of yeasts and fungi differ considerably in several properties from other plant sucrases.[4] By immunizing animals with pancreatin, M. Ascoli and Bonfanti[5] have succeeded in developing an antibody in the inactive serum of the rabbit acting specifically against pancreas diastase. They assume to have demonstrated that the serum amylases are specific for various kinds of starch, thus supplementing the explanation of the different characters of the salivary amylolysis described by Hamburger.[6] And they proceeded even to show that the anti-amylase of one animal acts upon the serum-amylase of another species in a different degree. In a paper[7] prepared under Professor Mendel's guidance, it was suggested that enzymes of plant origin act upon plant carbohydrates more readily than those of animal origin.

The influence of the serum upon the aspergillus sucrase and the sucrase of different origin was determined in the following four series of experiments:

a. The influence of normal serum upon aspergillus sucrase.

b. The influence of the anti-aspergillus serum upon aspergillus sucrase.

c. The influence of normal serum upon intestinal sucrase.

d. The influence of the anti-aspergillus serum upon intestinal sucrase.

From the results of several experiments in each series, it appears that normal rabbit serum, even when boiled or heated

[1] Cl. Bernard: *Leçons sur le Diabète*, Paris, p. 259, 1887.
[2] Cl. Bernard: cit. Schützenberger, *Intern. Wiss. Bibl.*, p. 259, 1876.
[3] *Amer. Journ. of Physiol.*, xiv, p. 239, 1905.
[4] Cf. Oppenheimer: *Die Fermente u. ihre Wirkungen*, p. 206, 1900.
[5] *Zeitschr. f. physiol. Chem.*, lxiii, p. 156, 1905.
[6] *Jahresber. f. d. ges. Med.*, 1871.
Saiki: this *Journal*, ii, p. 263, 1906.

at 65° for 30 minutes inhibits considerably the inversion of sucrose by the aspergillus extract, a result to be expected from the similar influence on the digestive action of inulase. The anti-aspergillus serum has a yet more powerful sucrase-inhibiting effect than has the normal serum, though this is not so pronounced as in the inulin experiments. When compared with the normal serum controls, inversion with intestinal sucrase is not influenced by the antiserum.

The greater inhibitory effects of the antiserum on the inulin digestions as contrasted with the results obtained below with sucrose, point to the existence of individual inulin-splitting and sucrose-inverting enzymes in the aspergillus. The experiments show, as indicated by the specificity of the anti-aspergillus serum, a difference between the intestinal and the aspergillus sucrase. For the differentiation of the sucrases, further confirmatory observations with a more active anti-aspergillus-sucrase are desirable, as well as a determination of the influence of the anti-intestinal sucrase on the sucrose-splitting enzyme contained in the mould. The observations might, also, be extended to include the sucrases of other species.

a. The Influence of Normal Serum and Heat Inactivated Normal Serum upon Aspergillus Sucrase.

EXPERIMENT XVII (SERUM FROM NORMAL RABBIT 8).

		CuO in 2 cc. gm.
	2 per cent sucrose solution,* 6.5 cc. + 5 per cent NaF solution, 1 cc.	
+ Serum	0.5 ⟶ 2 cc. + 1 per cent inulase solution 0.5 cc.	0.0151
+ "	0.5 ⟶ 2 " (boiled) + 1 " " " " 0.5 "	0.0141
+ "	0.5 ⟶ 2 " + 1 " " " " (boiled) 0.5 cc.	0.0012
+ "	0.5 ⟶ 2 " (boiled) + 1 " " " " 0.5 "	0.0023
+ Water	2 cc. + 1 per cent inulase solution, 0.5 cc.	0.0244
+ "	2.5 cc.	trace

Digestions kept at 38° for 20 hours.

EXPERIMENT XVIII (SERUM FROM NORMAL RABBIT 8).

		CuO in 1 cc. gm.
	2 per cent sucrose solution, 5 cc. + 5 per cent NaF solution, 1 cc.	
+ Serum	1 ⟶ 3 cc. (heated at 65° for 30 min.) + 1 per cent inulase solution, 1 cc.	0.0111
+ "	1 ⟶ 3 " (boiled) + 1 per cent inulase solution, 1 cc	0.0102
+ "	1 ⟶ 3 " (fresh) + 1 " " " " 1 "	0.0098

Digestions kept at 38° for 20 hours.

* The sucrose was purified by recrystallization from water.

b. The Influence of the Anti-aspergillus Serum upon Aspergillus Sucrase.

EXPERIMENT XIX (SERUM FROM IMMUNIZED RABBIT 2).

		CuO in 6 cc. gm.
	2 per cent sucrose solution, 6.5 cc. + 5 per cent NaF solution, 1 cc.	
+ Serum	0.5 ⟶ 1.5 cc. + 1 per cent inulase solution 1 cc	0.0171
+ "	0.5 ⟶ 1.5 " (boiled) + 1 " " " " 1 "	0.0250
+ "	0.5 ⟶ 1.5 " " + 1 " " " " 1 " (boiled)	0.0028

Digestions kept at 38° for 20 hours.

c. The Influence of Normal Serum upon Intestinal Sucrase.

EXPERIMENT XXII (SERUM FROM NORMAL RABBIT 9).

2 per cent sucrose solution, 5.5 cc. + 5 per cent NaF solution, 1 cc.		CuO in 2 cc. gm.
+ Serum, 1 ⟶ 3 cc	+ intestinal extract* 0.5 cc	0.0133
+ " 1 ⟶ 3 " (boiled)	+ " " 0.5 "	0.0137
+ " 1 ⟶ 3 " "	+ " " 0.5 " (boiled)	trace

Digestive Mixtures were kept at 38° for 20 hours.

*The fresh mucosa of a normal rabbit's small intestine was ground up with sand, and extracted with water containing 0.05 per cent Na_2CO_3 and 0.5 per cent chloroform, then filtered, and the clear filtrate employed for the intestinal sucrase experiments.

d. The Influence of the Anti-serum upon Intestinal Sucrase.

EXPERIMENT XXVIII (SERUM FROM IMMUNIZED RABBIT 2)

2 per cent sucrose solution, 5.5 cc. + 5 per cent NaF solution, 1 cc.		CuO in 2 cc. gm.
+ Serum, 1 ⟶ 3 cc.	+ intestinal extract 0.5 cc	0.0185
+ " 1 ⟶ 3 " (boiled)	+ " " 0.5 "	0.0190
+ " 1 ⟶ 3 " "	+ " " 0.5 " (boiled)	trace

Digestive mixtures kept at 38° for 20 hours.

CONCLUSIONS.

1. In normal rabbit's serum, neither an inulin-splitting enzyme nor an antibody of inulase occurs. Sucrase and its antibody are likewise not found.

2. The addition of serum induces an inhibition of the digestive action of inulase, independent of the presence of proteid or alkali. The hydrochloric acid hydrolysis is not inhibited by serum.

3. By subcutaneous injection of inulase an antibody for inulase can be produced in rabbit's serum.

4. The anti-serum exhibits different degrees of inhibitory action upon the inulin-digesting and sucrose-inverting activities, respectively, of inulase preparations. Presumably, therefore, the inulin-splitting action and the sucrase activity exist independently in the preparation of so-called "inulase" from *Aspergillus niger*.

5. Upon the intestinal-sucrase, the anti-serum exerts no noticeable influence, or at least very little under the conditions of these experiments.

I desire to express my obligation to Doctors Park and Gibson for the opportunity of conducting this investigation in their laboratories.

THE INFLUENCE OF ALCOHOL ON THE METABOLISM OF HEPATIC GLYCOGEN.[1]

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INTRODUCTORY.

Inquiries concerning the accumulation and transformation of glycogen in the liver have, within recent years, led investigators to study the influences exerted on these hepatic processes by various poisons when introduced in the body. Metallic compounds, also substances of the aliphatic and aromatic series as well as bacterial poisons, have been used. The results of such researches indicate that inorganic as well as certain organic poisons cause the removal of glycogen from the liver.

Thus Kaufholz[3] has shown that phosphorus poisoning in rabbits causes rapid transformation of glycogen in the liver. Koch[4] obtained similar results with corrosive sublimate. Kissel,[5] working in the same laboratory, corroborated the findings of Koch, and also made the interesting observation that the transformation of glycogen induced in the liver by the administration of corrosive sublimate may be inhibited by means of alcohol. Garnier and Lambert[6] stated that after the intravenous injection of sodium chloride the liver was freed from glycogen. Kriukoff[7]

[1] The results of some of the experiments have already been communicated in preliminary reports: *Proceedings of the Society for Experimental Biology and Medicine*, 1905–06, iii, p. 58; *Journal of the Am. Med. Assn.*, xlvii, p. 1467, 1906.

[2] Research Fellow of the Rockefeller Institute.

[3] Kaufholz: Dissertation, Würzburg, 1894.

[4] Koch: *ibid.*

[5] Kissel: *Centralbl. f. inn. Med.*, xvi, p. 613, 1895.

[6] Garnier and Lambert: *Compt. rend. de la soc. de biol.*, xlix, p. 617, 1897.

[7] Kriukoff: Dissertation, Moskau, 1902.

carried out a large number of experiments with various substances to test their action in this regard. He reported that after subcutaneous injection of arsenic, phosphorus, corrosive sublimate, anilin, phenyl hydrazine, pyrogallol, sulphuric acid, sodium hydroxide, or carbolic acid, glycogen disappeared from the liver in twenty-four hours. Alcohol had the same effect if injected subcutaneously at intervals for a long period.

In this connection the work of Drummond and Noel Paton[1] may be mentioned. These investigators found that in acute adrenalin poisoning in rabbits the liver glycogen was markedly diminished. The same results were obtained by Doyen and Kareff[2] with adrenalin chloride when they injected this substance into the portal vein. Likewise pilocarpine may, according to these investigators, favor glycogen transformation in the liver. Mohr's[3] studies with various gastrointestinal irritants, such as aloin, arsenious acid and croton oil have led to the same conclusion.

Claude Bernard[4] was the first to announce that during fever the glycogen in the livers of animals decreased even if nourishment was given. His observations were confirmed by Bouley.[5] May[6] carried out related experiments on dogs and rabbits. He also found that in fever hepatic glycogen diminishes. May stated that fifteen hours after feeding cane sugar to rabbits in which fever was induced by injecting pathogenic bacteria, 1.69 to 5.05 per cent of glycogen was obtained from the livers of such animals. In control rabbits, which received the same amount of cane sugar, the quantity of glycogen in the livers varied between 9.18 and 11.93 per cent. His results were even more marked when twenty-four hours were allowed to elapse after feeding 30 grams of glucose to rabbits in febrile condition. The liver removed at the end of this time contained an average amount of 0.42 per cent of glycogen. The controls contained 2.71 per

[1] Drummond and Paton: *Journ. of Physiol.*, xxi, p. 92, 1904.

[2] Doyen and Kareff: *Compt. rend. de la soc. de biol.*, lvi, p. 716, 1897.

[3] Mohr: Dissertation, Würzburg, 1894.

[4] Bernard, Claude, quoted by Roger: *Arch. de physiol. norm. et pathol.*, 5th series, vi, p. 64, 1894.

[5] Bouley: *ibid.*

[6] May: *Zeitschr. f. Biol.*, xxx, p. 48, 1894.

cent of glycogen. Ott[1] obtained similar results. He likewise induced infection in rabbits, by the method previously employed by May. Cane sugar was then given such rabbits and only those whose temperature was 40° C. were used for the experiments. Fifteen hours later the livers of these rabbits were removed and examined for glycogen. An average of 5.5 grams of glycogen was found in these livers, while almost double this quantity was found in the livers of the control animals.

That bacterial toxins hasten the disappearance of glycogen from the liver is also made probable by the observations of Luschi[2] which indicate that the glycogen of the livers of animals brought to a maximum of glycogen accumulation diminishes before the expiration of six hours after infection. Colla,[3] who studied the effect of infectious diseases on glycogen, found that glycogen disappears from the liver during tetanus, diphtheria, anthrax or pneumonia. He also examined the livers of a number of children who died of diphtheria. Four, ten or twelve hours after death the livers were free from glycogen. The results obtained by Hirsch and Rolly,[4] however, do not agree with those just mentioned. After inducing strychnine tetanus (which was preceded by seven days' fasting), 3 cc. of a twenty-four hour bouillon culture of *Bacillus coli communis* were injected into rabbits. Their livers as well as their muscles contained appreciable amounts of glycogen. In one rabbit 0.332 gram of glycogen was found in the liver, which weighed 45 grams. The livers of the control rabbits were free from glycogen.

That some substances, although not glycogen formers, may, nevertheless, favor the accumulation of glycogen in the liver has been indicated by experiments with antipyretics and narcotics. Lepine and Porteret[5] carried out a large number of experiments on guinea pigs with antipyrin, acetanilid, quinine sulphate and sodium salicylate. They found 20 per cent more glycogen in the livers of these animals than in the controls. The investigations of Nebelthau corroborated these results.

[1] Ott: *Deutsch. Arch. f. klin. Med.*, lxxi, p. 267, 1901.
[2] Luschi: *Jahresbericht für Thierchemie*, xxx, p. 449, 1900.
[3] Colla: *Archiv. Ital. de biologie*, xxvi, p. 120, 1896.
[4] Hirsch and Rolly: *Jahresbericht für Thierchemie*, xxxiii, p. 604, 1903.
[5] Lepine and Porteret: *Compt. rend. de l'acad. de sci.*, cvi, p. 1023, 1888.

After subcutaneous injection of kairin, antipyrin or quinine into hens on the fifth or seventh day of fasting, he found 1.81 per cent to 3.66 per cent of glycogen in the livers. His work with sulphonal, urethane, chloralamid and paraldehyde, injected on the seventh day into fasting hens, likewise indicated the accumulation of glycogen in the livers. That paraldehyde exerts a similar effect in rabbits was shown by Nebelthau[1] and Cremer.[2] Nebelthau[3] also carried out a series of observations on the effects of ether, chloroform and alcohol on the metabolism of hepatic glycogen in hens. The results he obtained with these substances led him to the conclusion that they favor the accumulation of glycogen in the liver, although in his experiments with alcohol he obtained positive results in only four out of eleven experiments.

The discordant results obtained with alcohol by Nebelthau[4] and Kriukoff[5] furnished the indication for the present study of the action of that substance on the metabolism of hepatic glycogen. The determination of the action of alcohol in this regard is especially important also because clinicians frequently prescribe its internal administration in infectious diseases. Colla[6] maintained that resistance to bacterial invasion varies with the amount of glycogen in the liver. Although his results were not corroborated by the observations of Luschi,[7] the truth of this contention by Colla is made probable by the work of Roger,[8] who claimed that the glycogen seems to be an important factor in the liver in reducing the toxicity of alkaloids and other poisons of organic nature when these enter the circulation. The conclusions of Roger were disputed by Vamossy.[9] The evidence, however, which Vamossy brought forward against the view of Roger is not convincing.

[1] Nebelthau: *Zeitschr. f. Biol.*, xxviii, p. 138, 1891.
[2] Cremer: *Ergebnisse der Physiologie*, i, p. 876, 1902.
[3] Nebelthau: *loc. cit.*
[4] Nebelthau: *loc. cit.*
[5] Kriukoff: *loc. cit.*
[6] Colla: *loc. cit.*
[7] Luschi: *loc. cit.*
[8] Roger: *Centralbl. f. klinische Medizin*, ix, p. 5, 1888.
[9] Vamossy: *Archives internationales de pharmaco-dynamie et de therapie*, xiii, p. 208, 1904.

EXPERIMENTAL.

Methods.—The experiments were carried out on full grown healthy rabbits, between the months of December and May. Throughout the experimental period each animal was kept in a metallic cage provided with a wire net bottom and drip pan to allow drainage of the urine. The room was maintained at a practically uniform temperature since, as Lüthje,[1] and later Amalgia and Embden[2] have shown, the metabolism of carbohydrates is influenced by the temperature of the surrounding atmosphere. Before an experiment the animals were kept under observation for several days in the cages. If failure of adaptation to the new condition was manifested by any of these rabbits they were rejected. In this way some assurance was obtained that the subject of each experiment was normal.

When liver was subjected to analysis, the following procedure was always followed: The animal was quickly killed; its liver was rapidly removed and weighed and placed at once in hot 60 per cent potassium hydroxid solution. Glycogen in the livers was isolated by the shorter method of Pflüger.[3] The amount of glucose obtained from the glycogen by hydrolysis was determined by Allihn's method. Later in the course of the investigation, for reasons of economy of time, the amounts of copper thrown down by reduction were determined volumetrically by the iodin method. On account of the sharp end point given by the starch iodin reaction, the latter process was preferred to the cyanide method that is recommended by some investigators for the determination of copper.

The introduction of alcohol or glucose into the body was made *per os* through a stomach tube.

Series I. On fasting rabbits, with small preliminary accumulations of hepatic glycogen. The experiments of several workers, which showed the accumulation of glycogen in the liver after the administration of various narcotics and antipyretics, as well as the observations of Nebelthau[4] on hens which indicated

[1] Lüthje: *Beiträge zur chemischen Physiologie und Pathologie*, vii, p. 309, 1906.

[2] Amalgia and Embden: *ibid.*, p. 310.

[3] Pflüger: *Archiv für die ges. Physiol.*, xciii, p. 163, 1902.

[4] Nebelthau: *loc. cit.*

similar results in some cases with alcohol, suggested the advisability of carrying out similar experiments with alcohol on other animals. The possible synthesis of glycogen from alcohol was also thought of, for the work of Goddard[1] on dogs has shown that when large doses of alcohol are given, an appreciable amount of aldehyde may be produced in the blood. Again the high calorific value of alcohol and its ready oxidation in the body led to the expectation that even if alcohol failed to induce an accumulation of glycogen in the liver, its hepatic depletion commonly observed during fasting might, under the influence of alcohol, be reduced or perhaps even entirely inhibited. Experiments were therefore carried out on fasting rabbits, which were given 10 cc. of 30 per cent alcohol per kilo daily for four or five days.

EXPERIMENT 1A, FEMALE RABBIT.

Nov.	20,	2	p. m.	Weight	1180	grams.	Received	10 cc.	30	per cent	alcohol	per kilo.
"	21,	4	"	"	1180	"	"	10 "	30	"	"	"
"	22,	2.30	"	"	1120	"	"	10 "	30	"	"	"

Nov. 22, 3.30 p. m. The rabbit was killed. The weight of the liver was 48 grams. Analysis failed to show the presence ofglycogen.

EXPERIMENT 2A, WHITE FEMALE RABBIT.

Nov.	20,	2	p. m.	Weight	1280	grams.	Received	10 cc.	30	per cent	alcohol	per kilo.
"	21,	4	"	"	1210	"	"	10 "	30	"	"	"
"	22,	2.30	"	"	1170	"	"	10 "	30	"	"	"
"	23,	10	a. m.	"	1100	"	"	10 "	30	"	"	"

Nov. 23, 10.30 a. m. The rabbit appeared to be exhausted and dying. She was killed soon afterwards. The weight of the liver was 35 grams. In this rabbit also the liver was free from glycogen.

As controls were used two female rabbits (1 and 2, Table I) to which water instead of alcohol was administered by mouth, through a stomach tube on four successive days. At the end of this period they were killed and the content of glycogen in their livers determined.

This analysis, Table I, as well as that for both the alcoholized rabbits, Table II, failed to show the presence of glyco en in the liver of any one of these animals.

Our finding indicates, therefore, that alcohol, in the amounts given, does not favor the accumulation of glycogen in the livers of fasting rabbits; neither was there any manifestation of the sparing effect of fats or carbohydrates commonly ascribed to alcohol.

[1] Goddard: *Lancet*, ii, p. 1132, 1904.

Series II. On fasting rabbits, with normal preliminary accumulations of hepatic glycogen. Since the foregoing general result might be due to the presence of only relatively low proportions of glycogen in the livers of these animals, which were fed on hay, oats and cabbage, previous to the alcohol period, a series of experiments was carried out in which carrots were fed in large quantities during the fore period of three days in order to induce accumulation of normal amounts of glycogen in the livers of the animals selected. Alcohol (approximately 10 cc. of 30 per cent alcohol per kilo) was then administered daily for four, five or six days.

EXPERIMENT 5A, WHITE RABBIT.

Dec.	13	Weight	1300	grams.	Received	15	cc.	30	per cent	alcohol.
"	14	"	1260	"	"	13	"	30	"	"
"	15	"	1210	"	"	13	"	30	"	"
"	16	"	1180	"	"	25	"	30	"	"
"	17	"	1070	"	"	12	"	30	"	"
"	18	"	1100	"	"	12	"	30	"	"

Dec. 18. The rabbit was killed. The weight of the liver was 44 grams. The amount of glucose obtained by hydrolysis of glycogen was 0.92 per cent of the fresh tissue.

EXPERIMENT 6A, WHITE RABBIT.

Dec.	12	Weight	1580	grams.	Received	16	cc.	30	per cent	alcohol.
"	13	"	1540	"	"	15	"	30	"	"
"	14	"	1500	"	"	15	"	30	"	"
"	15	"	1440	"	"	15	"	30	"	"
"	16	"	1400	"	"	25	"	30	"	"
"	17	"	1300	"	"	13	"	30	"	"
"	18	"	1300	"	"	13	"	30	"	"

Dec. 18. The rabbit was killed. The weight of the liver was 48 grams. The amount of glucose obtained by hydrolysis of glycogen was 0.31 per cent of the fresh tissue.

EXPERIMENT 9A, FEMALE RABBIT.

Dec.	24	Weight	1520	grams.	Received	15	cc.	30	per cent	alcohol.
"	25	"	1420	"	"	15	"	30	"	"
"	26	"	1410	"	"	15	"	30	"	"
"	27	"	1350	"	"	13	"	30	"	"
"	28	"	1300	"	"	13	"	30	"	"
"	29	"	1280	"	"	15	"	30	"	"
"	30	"	1220	"	"	19	"	30	"	"

Dec. 30, 2.40 p. m. The rabbit was killed. The weight of the liver was 47 grams. Only a trace of glucose was obtained.

EXPERIMENT 7A, MALE RABBIT.

Dec.	24	Weight	1670 grams.	Received	17 cc.	30 per cent alcohol.			
"	25	"	1700 "	"	17 "	30	"	"	

Several hours after it received the last dose, the rabbit escaped from the cage and was found eating hay and oats. The experiment was then continued, as follows:

Dec.	26	Weight	1620 grams.	Received	17 cc.	30 per cent alcohol.			
"	27	"	1580 "	"	17 "	30	"	"	
"	28	"	1480 "	"	15 "	30	"	"	
"	29	"	1530 "	"	20 "	30	"	"	
"	30	"	1470 "	"	19 "	30	"	"	
"	31	"	1500 "	"	15 "	30	"	"	

Dec. 31, 5 p. m. The rabbit was killed. The weight of the liver was 53 grams. The amount of glucose obtained by hydrolysis of the glycogen was 0.09 per cent of the fresh tissue.

EXPERIMENT 9B, GRAY FEMALE RABBIT.

Dec.	24	Weight	1410 grams.	Received	15 cc.	30 per cent alcohol.			
"	25	"	1450 "	"	15 "	30	"	"	

(At this point there was a fasting period of the same length and for identical reasons as that for rabbit 7a.)

Dec.	26	Weight	1420 grams.	Received	15 cc.	30 per cent alcohol.			
"	27	"	1350 "	"	14 "	30	"	"	
"	28	"	1370 "	"	14 "	30	"	"	
"	29	"	1350 "	"	15 "	30	"	"	
"	30	"	1300 "	"	20 "	30	"	"	
"	31	"	1270 "	"	14 "	30	"	"	

Dec. 31, 5 p. m. The rabbit was killed. The weight of the liver was 43 grams. Only a trace of glucose was obtained.

EXPERIMENT 10A, GRAY RABBIT.

Jan.	1	Weight	1830 grams.	Received	18 cc.	30 per cent alcohol.			
"	2	"	1690 "	"	17 "	30	"	"	
"	3	"	1540 "	"	15 "	30	"	"	
"	4	"	1510 "	"	15 "	33	"	"	
"	5	"	1470 "	"	15 "	33	"	"	

Jan. 5. The rabbit was killed. The weight of the liver was 53 grams. The amount of glucose obtained by hydrolysis of the glycogen was 0.02 per cent of the fresh tissue.

EXPERIMENT 11A, GRAY FEMALE RABBIT.

Jan.	1	Weight	1700 grams.	Received	18 cc.	33 per cent alcohol.			
"	2	"	1560 "	"	16 "	33	"	"	
"	3	"	1460 "	"	15 "	33	"	"	
"	4	"	1450 "	"	15 "	33	"	"	
"	5	"	1350 "	"	14 "	33	"	"	

Jan. 5. The rabbit was killed. The weight of the liver was 54 grams The amount of glucose obtained by hydrolysis of the glycogen was 0.16 per cent of the fresh tissue.

The results obtained in the experiments of this series, which are also shown in Table II, likewise failed to give evidence of any inhibitory action of alcohol on the depletion of hepatic glycogen. Of the three rabbits which received alcohol daily for *six* days, the liver in one contained less than 0.1 per cent of glycogen; in each of the livers of the other two rabbits only traces of glycogen were present at the conclusion of the experiments. In one rabbit, 8, Table I, which was used as a control and was given water by mouth through a stomach tube for the same length of time after preliminary feeding with carrots, the amount of glycogen in the liver was a little more than 0.04 per cent. In this connection it may be pointed out that the livers of two normal rabbits, kept on a diet of carrots for three days, contained 3 to 4 per cent of glycogen at the end of that time.

In two experiments alcohol was given during a period of *five* days. Appreciably larger amounts of glycogen were found in the livers of these rabbits than in the livers of the controls (see Tables 1 and 2, p. 416), which would seem to indicate that alcohol caused retarded transformation of glycogen. This was improbable, however, in the light of the results of Experiments 10a and 11a. In the latter experiments, very small amounts of glycogen were found in the livers of these rabbits, which received alcohol on four days after the usual preliminary feeding of carrots. It was thought, however, that possibly larger quantities of alcohol and consequently an increase in the number of calories might spare the hepatic glycogen. Two experiments were carried out to test this suggestion (Series III).

Series III. Same as Series II, with larger doses of alcohol.

EXPERIMENT 17A, WHITE RABBIT.

Fasted for six days, was then fed carrots on four days (Jan. 30 to Feb. 3.)

Feb.	3	Weight 1800 grams.	Received	20 cc.	30	per cent	alcohol.
"	4	3 p.m.	"	20 "	60	"	"
"	5	11.30 a.m.	"	10 "	60	"	"
"	5	3 "	"	10 "	60	"	"
"	6	10 a.m.	"	10 "	60	"	"
"	6	3 p.m.	"	12 "	60	"	"
"	7	12 noon	"	13 "	60	"	"

At 3.30 p. m. the rabbit was found dead, but was still warm. The weight of the liver was 66 grams. A qualitative test failed to show the presence of glycogen in the liver.

EXPERIMENT 18A, GRAY RABBIT.

Fasted six days. Carrots were given on four days. (Jan. 30 to Feb. 3).

Feb.	3	Weight 1130 grams.	Received	15 cc.	30 per cent alcohol.			
"	4	3 p.m.	"	12	"	60	"	"
"	5	11.30 a.m.	"	8	"	60	"	"
"	5	2.30 p.m.	"	7	"	60	"	"
"	6	10 a.m.	"	8	"	60	"	"
"	6	3 p.m.	"	8	"	60	"	"
"	7	12 noon	"	10	"	60	"	"
"	7	8.30 p.m.	"	10	"	60	"	"

Feb. 7, 10.30 p. m. The rabbit was killed. The weight of the liver was 45 grams. As in the preceding experiment the liver was free from glycogen.

The results of analysis in these experiments (Series III) likewise indicate that even somewhat larger quantities of alcohol than those of the previous experiments failed to inhibit the disappearance of glycogen from the livers of fasting rabbits, this organ in each rabbit having been found free from glycogen. The question whether alcohol may accelerate the transformation of glycogen in the liver now presented itself with special force.

Series IV. Does alcohol accelerate the transformation of hepatic glycogen. To answer this question various amounts of glucose were given to rabbits by mouth through a stomach tube. Alcohol was administered either immediately after the glucose was given or, as in some experiments, a few hours were allowed to elapse between the last feeding of glucose and the succeeding dose of alcohol. In some experiments two doses of alcohol were administered at intervals of eighteen to twenty-four hours. In one experiment (28a), only one dose was given while another rabbit (29a) received three doses of alcohol. As controls I used rabbits which were fed varying amounts of glucose. The rabbits were killed at the end of different periods following the administration of glucose.

Experiment 24a, white rabbit. Fasted 6 days. Weight, 1800 grams.

March 19, 10 grams of glucose dissolved in water were given by mouth. Immediately afterward the animal received 10 cc. of 60 per cent alcohol.

March 20, 10 grams of glucose were fed, then 15 cc. of 60 per cent alcohol were given.

About sixteen hours later the rabbit was killed. The weight of the liver was 52 grams. The amount of glucose obtained by hydrolysis of glycogen was 1.9 per cent of the fresh tissue.

Experiment 24b, black rabbit. Weight, 1850 grams. Fasted 6 days.

March 19, 10 grams of glucose given by mouth.

March 20, 10 grams glucose given by mouth.

Sixteen hours later the rabbit was killed. The weight of the liver was 51 grams. The amount of glucose obtained by hydrolysis of glycogen was 4 per cent of the fresh tissue.

Experiment 25a, rabbit. Weight, 1550 grams. Fasted 6 days.

May 9, 3.30 p.m. Eight grams of glucose given by mouth.

May 9, 10 p.m. Received 15 cc. of 60 per cent alcohol.

May 10, 11 a.m. Weight, 1500 grams. Received 15 cc. of 60 per cent alcohol.

The rabbit was killed May 11 at 3 p.m. About 15 minutes after alcohol had been given, symptoms of intoxication appeared which lasted several hours. At the time of death, the rabbit looked normal. No glycogen was present in the liver.

Experiment 25. Rabbit fasted 6 days. May 10, 10 a.m. Weight 1850 grams. Nine grams of glucose were given by mouth. May 10, 3 p.m. Water was given by mouth. The rabbit was killed May 11 at 7 p.m. The weight of the liver was 33 grams. The amount of glucose obtained by hydrolysis of glycogen was 1.23 per cent of the fresh tissue.

Experiment 26a. Weight of rabbit, 1450 grams. Fasted 6 days.

May 9, 3.30 p.m. Seven grams of glucose, dissolved in water, were given by mouth.

May 9, 10 p.m. Fifteen cc. of 60 per cent alcohol were given by mouth.

May 10, 11 a.m. Weight, 1300 grams. Received 13 cc. of 60 per cent alcohol.

Shortly afterward, the rabbit manifested symptoms of severe intoxication which continued all day. While still under the influence of alcohol, May 10, 7 p.m., the rabbit was killed. The amount of glucose obtained by hydrolysis of the glycogen was 3.33 per cent of the fresh tissue.

Experiment 27a. Rabbit fasted 6 days.

May 9, 4 p.m. Weight, 1200 grams. Six grams of glucose were given by mouth.

May 9, 10 p.m. Received 15 cc. of 60 per cent alcohol.

May 10, 11 a.m. Weight, 1100 grams. Received 11 cc. of 60 per cent alcohol.

May 10, 7 p.m. The rabbit was killed. Signs of intoxication developed ten minutes after the administration of alcohol. At the time of death the rabbit looked normal. The weight of the liver was 36 grams. The liver of this rabbit was free from glycogen.

Experiment 28. The rabbit fasted 6 days.

May 27, 6. p.m. Weight, 1450 grams. Fifteen grams of glucose dissolved in water were given by mouth.

May 28, 12 noon. Weight, 1450 grams. Fifteen grams of glucose were given as before.

May 30. The rabbit struggled a good deal when he was fastened on the holder.

May 30, 6 p.m. Killed. The weight of the liver was 30 grams. The amount of glucose obtained by hydrolysis of the glycogen was 0.11 per cent of the fresh tissue.

Experiment 29a. Rabbit fasted 6 days.

May 27, 6 p.m. Weight, 1450 grams. Fifteen grams of glucose were given by mouth.

May 28. Weight, 1450 grams. Fifteen grams of glucose were given as before.

May 28, 6.15 p.m. Received 15 cc. of 60 per cent alcohol.

May 29, 5 p.m. Received 10 cc. of 60 per cent alcohol.

May 30, 11 a.m. Weight, 1450 grams. Received 10 cc. of 60 per cent alcohol.

May 30, 4.30 p.m. The rabbit was killed while apparently still under the influence of alcohol. The weight of the liver was 44 grams. Analysis did not show the presence of glycogen.

Experiment 30a. Rabbit fasted 6 days.

May 27, 6 p.m. Weight, 1270 grams. Received 13 grams of glucose dissolved in water.

May 28, 12.15 p.m. The same amount of glucose was given.

May 29, 5.30 p.m. Received 12 cc. of 60 per cent alcohol.

May 30, 11 a.m. Received 15 cc. of 60 per cent alcohol.

May 30, 4.30 p.m. The rabbit was killed while still under the influence of alcohol. This liver was likewise free from glycogen.

Experiment 31a. Rabbit fasted 6 days.

May 27, 6. p.m. Weight of rabbit, 1800 grams. Eighteen grams of glucose were given. This was repeated next day. Fifty-two hours later the rabbit was killed. The liver was removed and treated as in the other experiments. The weight of the liver was 28 grams. The amount of glucose obtained from the glycogen was 1.24 per cent of the fresh tissue.

Experiment 28a. Rabbit fasted 6 days. Weight, 1230 grams.

May 27 and 28. Thirteen grams of glucose were administered as in previous experiments.

May 28, 6 p.m. Received 13 cc. of 60 per cent alcohol.

May 29, 6 p.m. The rabbit was found in a dying condition. It was then killed. The liver was removed immediately and subjected to analysis by the usual method. Weight, 45 grams. The amount of glucose obtained from the glycogen was 1.24 per cent of the fresh tissue.

The analytic results given in the table (III) show with one exception (No. 26a) a marked diminution in the glycogen of the livers of the alcoholized rabbits as compared with the controls. Thus, sixteen hours after the last feeding of glucose the total amount of glycogen obtained was 3.7 per cent in the control (24), while in the rabbit given alcohol (24a), which received the same amount of glucose (20 grams in twenty-four hours), the quantity

of glycogen found in the liver was 1.76 per cent, less than half that found in the control.

In the next group each rabbit was given a single dose of 5 to 9 grams of glucose per kilo. They were killed twenty-eight hours later. Excepting rabbit 26a this series showed in a striking manner the action of alcohol in hastening the transformation of hepatic glycogen, for, whereas the amount of glycogen in the controls was 1.14 per cent (25) and 2.3 per cent (26), the livers of the two corresponding alcoholized rabbits were free from glycogen. The administration of alcohol after feeding much larger quantities of glucose (20 gm. per kilo in twenty-four hours) was accompanied by similar results when the rabbits were killed in from fifty-two to fifty-four hours after they received the final dose of glucose. In the two controls of this group the amount of glycogen obtained was 0.11 per cent in one (28), and 1.14 per cent (31a) in the other, whereas the livers of the two alcohol rabbits were free from glycogen. In another experiment of this group (28a) the rabbit received the same amount of glucose per kilo in twenty-four hours, but, as he was in a dying condition, apparently from the effects of alcohol, he was killed thirty hours after he had received the final dose of glucose. The liver of this rabbit contained only 1.33 per cent of glycogen, which is only 0.2 per cent more than that found in the control rabbit (25) and about 1 per cent less than in rabbit 26, which received one-fourth the amount of glucose per kilo fed to rabbit 28a.

The discrepancy between the results obtained for rabbits 26a, and in the other alcohol experiments of the same series, may be explained by the following observations: It was noted that in this rabbit symptoms of severe alcohol intoxication (which set in soon after the second and final dose of alcohol was given) persisted eight hours, while the other rabbits of this group similarly treated behaved at the end of this time as if they had recovered completely from the effects of alcohol intoxication. This difference in the reaction to alcohol is suggestive of the possibility that during the stage of profound alcohol narcosis, glycogen metabolism is not affected. Perhaps it is only when recovery from narcosis is practically complete that metabolism of glycogen is resumed.

In the two alcohol rabbits 29a and 30a, each of which was killed in a state of deep intoxication, this supposition apparently

TABLE I. AMOUNTS OF GLYCOGEN IN THE LIVERS OF CONTROL RABBITS.

Exp. No.	Rabbit.	Liver.	Food before fasting.	Fasting period.	Treatment during fasting.	Hepatic glycogen; per cent of the fresh tissue calculated from glucose.
	gms.	gms.		days		
1	820	22	C. H. O.*	4	Water given	none
2	1320	41	"	5	by stomach	"
5	1230	27	Carrots 3 days.	5	tube.	0.139
6	970	22	"	5		0.148
8	1370	42	"	6		0.043
10	1265	38	"	4		0.127
11	1470	53	"	4		none

TABLE II. AMOUNTS OF GLYCOGEN IN THE LIVERS OF FASTING RABBITS AFTER ADMINISTRATION OF ALCOHOL.

Exp. No.	Rabbit.	Liver.	Food before fasting.	Fasting period.	Alcohol per kilo daily.	Hepatic glycogen; per cent of the fresh tissue calculated from glucose.
	gms.	gms.		days	30% in cc.	
1a	1120	48	C. H. O.*	4	10	none
2a	1100	35	"	5	10	"
5a	1100	44	Carrots 3 days	5	10	0.84
6a	1300	48	"	5	10	0.28
9a	1280	47	"	6	10	trace
7a	1500	53	"	6	10	0.083
9b	1270	43	"	6	10	trace
10a	1470	53	"	4	10	0.018
11a	1350	54	"	4	10	0.148
3a	800	35	C. H. O.	4	12	trace
					60 % in cc.	
17a	1800	66	Carrots 3 days	3½	12	none
18a	1130	45	"	4	15	none

* C. H. O.—Cabbage, hay, oats.

was not borne out by the results of the analysis. As shown in the table, their livers did not contain any glycogen. The protocols show, however, that the intervals between the two successive doses of alcohol in each case were eighteen or twenty-four hours, thus giving sufficient time for recovery from its intoxicating effect. In rabbit 26a, the interval was only twelve hours, which may account for the different results obtained.

TABLE III. SHOWING THE EFFECTS OF COMPARATIVELY LARGE QUANTITIES OF ALCOHOL ON THE METABOLISM OF HEPATIC GLYCOGEN.

Exp. No.	Rabbit.	Liver.	Glucose fed per kilo in 24 hours.	60% alcohol per kilo daily.	No. hours between last feeding of glucose and death.	Hepatic glycogen; per cent of the fresh tissue calculated from glucose.
	gms.	gms.	gms.	cc.		
24	1850	51	20	0	16	3.70
24a	1800	52	20	25	16	1.76
25	1850	33	9	0	28	1.14
25a	1550	48	8	30	28	
26a	1450	58	7	28	28	3.08
26	1120	41	5½	0	28	2.30
27a	1200	36	6	26	28	
28	1450	30	29	0	54	0.11
29a	1450	44	29	35	52	
30a	1270	41	26	27	52	
31a	1800	28	36	0	52	1.14
28a	1230	45	25	13	30	1.33
21a	1050	41	32	20	12	8.65
23a	815	35	25	12	7	9.17

The suggestion that alcohol does not hasten the transformation of glycogen of the liver during the stage of profound narcosis gains further support from the results of experiments 21a and 23a, in which large quantities of glucose were followed by alcohol (shortly after, in one experiment, or three hours later, in another). The amounts of glycogen found six to twelve hours after the final dose of glucose had been given were 9.17 per cent and 8.65 per cent, respectively.

It is to be noted that in both these experiments the rabbits were killed from six to eight hours after receiving large amounts of alcohol in proportion to body weight, that is, before the stage of intoxication passed off. The conclusion seems to be justified, therefore, that large quantities of alcohol may hasten the process by which glycogen is made to disappear from the liver and that it apparently exerts this action only after the stage of intoxication has been passed. This mode of action of alcohol might explain the discordant results of Nebelthau's experiments, some of which showed disappearance of hepatic glycogen, while others indicated the presence of considerable amounts, after administration of alcohol. It may in the same way also account

for the different results obtained by Nebelthau and Kriukoff, whose work was referred to on p. 406.

Objection to this conclusion may be raised on the ground that there is a wide range of variation in the proportion of glycogen found in the livers of the control rabbits. Thus in experiment 28a, only 0.11 per cent was obtained, while in 31a, the presence of 1.14 per cent of glycogen was shown. This difference may possibly have been due to the activity of rabbit 28a, when it was tied on the holder before it was killed. The other rabbits did not behave in this way. Again, in experiments 25 and 26 (two control rabbits) there was a difference of 50 per cent in the liver glycogen found; as great a difference as there was between the alcohol rabbit in experiment 24a, and the corresponding control. The wide variation in the amount of hepatic glycogen of these two normal rabbits does not invalidate the above conclusions, since in the alcoholized rabbits of the same group (25a and 27a), which received as much glucose per kilo as the controls, and were killed at the same time after feeding glucose, the livers were free from glycogen. Moreover, the difference between the amounts obtained from the two normal rabbits may possibly be accounted for by a difference in muscular activity. It is of importance to note, in this connection that, in the alcoholized rabbits, intoxication and consequent loss of muscular power set in about 15 minutes or sometimes even earlier after alcohol was administered, which would tend to inhibit rather than hasten the amylolysis.

That other toxic substances may exert an action on hepatic glycogen comparable to that here attributed to alcohol was shown by Roger.[1] He was the first to find that anthrax has no effect on glycogen during the first stage of infection, but later accelerates its disappearance from the liver.

I am very much indebted to Prof. William J. Gies for valuable suggestions received in the course of this investigation.

[1] Roger: *Arch. de physiol. norm. et pathol.*, 5th series, vi, p. 64, 1894.

ON THE PRODUCTION OF PHENOLIC ACIDS BY THE OXIDATION WITH HYDROGEN PEROXIDE OF THE AMMONIUM SALTS OF BENZOIC ACID AND ITS DERIVATIVES, WITH SOME REMARKS ON THE MODE OF FORMATION OF PHENOLIC SUBSTANCES IN THE ORGANISM.

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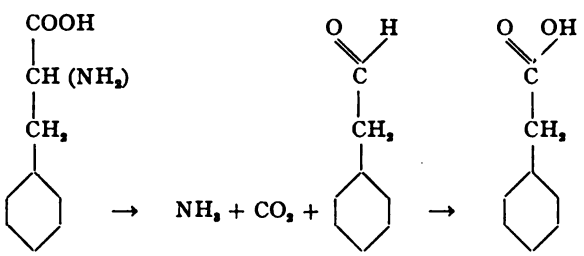
While continuing the investigation of the action of hydrogen peroxide upon various amido-acids,[1] the attempt was made to isolate the products of the oxidation of phenylalanin. Judging from analogy with aliphatic amido-acids, such as alanin which yields acetaldehyde, acetic acid, ammonia and carbon dioxide, it was anticipated that the products would be phenylacetaldehyde, phenylacetic acid, ammonia and carbon dioxide.

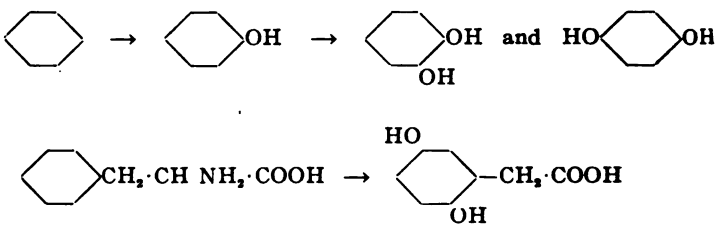


Although undoubted indications of the formation of small quantities of phenylacetaldehyde were obtained, showing that the reaction, at least in part, had proceeded as was anticipated, it was found that acid products were formed at the same time,

[1] Dakin: This *Journal*, i, p. 171, 1906.

which were certainly not exclusively composed of phenylacetic acid.

Subsequent investigation showed that phenolic acids were present, for the product gave a violet-blue coloration with ferric chloride and also a strong reaction with Millon's reagent, thus proving that oxidation had occurred in the nucleus as well as in the side-chain. The fact that it had been possible to effect the introduction of one or more hydroxyl groups into an aromatic nucleus by oxidation of an amido-acid at the ordinary temperature at once suggested that the reaction had some biological significance, for it is known that similar changes occur in the animal organism, as exemplified in the formation of homogentisic acid from phenylalanin and tyrosin in alkaptonuria and in the conversion by the organism of benzene into phenol, pyrocatechin and hydroquinone.[1] The formation of adrenalin, which contains the catechol nucleus from any of the known aromatic substances ordinarily supplied to the animal organism (phenylalanin, tyrosin and tryptophan) would necessitate similar oxidations:



A preliminary attempt to determine more exactly the nature of the products formed by the oxidation of phenylalanin showed that the reaction was a somewhat complicated one, in which the first stage consisted in the formation of phenylacetaldehyde (phenylacetic acid) ammonia and carbon dioxide. In order to obtain information as to the further oxidation products, it was decided to study the action of peroxide upon simpler nitrogen-free acids. It was found that hydrogen peroxide could effect the introduction of hydroxyl groups into the benzene nucleus of

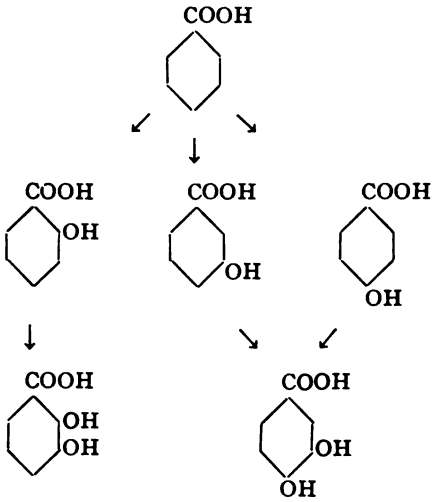
[1] Schultzen and Naunyn: *Arch. f. Physiol.*, p. 349, 1867; Baumann and Preusse: *Zeitschr. f. physiol. Chem.*, iii, p. 156; vi, p. 190.

a series of aromatic acids, such as benzoic acid, phenylacetic, phenylpropionic and their substitution derivatives. The question of the oxidation products of phenylacetic and phenylpropionic acids will be discussed in a future paper. The present communication consists in an account of the oxidation of benzoic acid and some of its substitution derivatives.

It was found that if benzoic acid be dissolved in water containing hydrogen peroxide and a slight excess of ammonia and the mixture allowed to stand at the ordinary temperature of the air, it was easy to demonstrate the formation of salicylic acid. The salicylic acid may be detected by simply acidifying, extracting with ether and applying the ferric chloride or other test to an aqueous solution of the ethereal extract. Further investigation showed that salicylic acid was not the only product of the reaction but para- and meta-oxybenzoic acids were formed at the same time and in not widely differing amounts.

The salicylic acid was separated from the other oxyacids by means of its solubility in chloroform. The free acid was obtained from the sparingly soluble basic calcium salt and was further identified by conversion into tribromophenol bromide, melting point 131°, and tribromophenol, melting point 93–95°. The meta-oxybenzoic acid on treatment with bromide water, gave 2,4,6-tribromo-3-oxybenzoic, melting point 145–146°, while the para-oxybenzoic acid was separated in the form of its very sparingly soluble basic barium salt, from which the pure acid was easily obtained, melting at 210–211°. It was also converted into tribromophenol bromide and tribromophenol. The details of the separation will be found in the experimental part of this paper.

Further investigation showed that a second hydroxyl group could be introduced into the mono-oxybenzoic acids by treatment of their ammonium salts with hydrogen peroxide. In each case the second hydroxyl took up a position ortho to the first (OH) group. The 2,3-dioxybenzoic acid and 3,4-dioxybenzoic acid were both obtained in the pure crystalline state, melting at 202° and 199–200° respectively. The following scheme represents the progressive oxidation of benzoic acid:

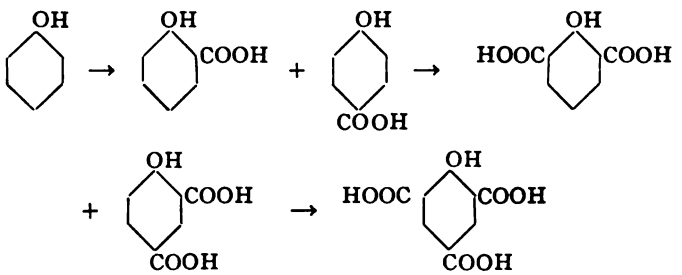


The action of the hydrogen peroxide in effecting the introduction of hydroxyl groups into the aromatic nucleus of benzoic acid and its derivatives seems to be a general reaction, for experiments carried out with a series of chlor-, brom-, nitro-, dinitro-, and amido-benzoic acids, all resulted in the production of phenolic acids, although the yields appear to be in all cases very small.

The simultaneous production of the three isomeric oxybenzoic acids is of some interest from several points of view. In the first place the simultaneous production in considerable amounts of ortho, meta and para derivatives is not very common. It might be expected that the direct substitution of a benzene derivative, C_6H_5X, where X is an acidic group, such as COOH, would result in the formation of a meta derivative,[1] and although this is true for most substituents, yet in the case of the direct introduction of an (OH) group the tendency to form ortho and para derivatives is at least as strong. In the case of the dihydroxy-acids the position of the second entering (OH) group appears to be governed mainly by the position of that already present in the ring, the second (OH) going into the ortho (and para) position to the first. The reaction furnishes an additional

[1] Hübner: *Ber. d. deutsch. chem. Gesellsch.*, viii, p. 873; Nölting: *ibid.*, ix, p. 1797.

exception to Brown and Gibson's generalization,[1] which states that if a benzene substituent HX is convertible by direct oxidation into a compound XOH, then substitution will result in the formation of meta derivatives. If, on the other hand, direct oxidation is not practicable, ortho and para substitution derivatives will be produced. It is interesting to note that the reverse change to that under consideration, namely, the introduction of carboxyl groups into phenol, results in the formation of acids in which the carboxyl takes up a position ortho and para to the hydroxyl group.



Excluding reactions taking place in the living organism, we have thus far been able to find only two other cases of the direct introduction, by oxidation, of hydroxyl groups into an aromatic acid[2] not already containing hydroxyl groups. Both of these are reactions occurring at high temperatures and therefore possessing limited biological significance. Thus Etting showed that copper benzoate on heating to 275° yields some copper salicylate,[3] while Barth and Schreder determined the pressure of oxybenzoic acids among the products of the fusion of benzoic acid with potash.[4]

A few instances are recorded of the direct introduction of the (OH) group into the ring of aromatic hydrocarbons. Thus benzene has been directly oxidized to phenol by means of ozone,[5]

[1] *Journ. Chem. Soc.*, lxi, p. 368.
[2] *Ann. d. Chem.*, liii, pp. 88, 91.
[3] *Monatsh. f. chem.*, iii, p. 799.
[4] Nencki and Giacosa: *Zeitschr. f. physiol. Chem.*, iv, p. 339.
[5] See, however, the action of potassium persulphate upon hydroxycids referred to later.

hydrogen peroxide,[1] by air in the presence of water and palladium-hydrogen,[2] or copper or iron salts[3]—reactions which probably depend on the production of hydrogen peroxide—also by the action of sunlight in the presence of caustic soda[4] and finally by the combined action of oxygen and aluminum chloride.[5]

The production of phenol by the oxidation of benzene with peroxide of hydrogen has been questioned by Kingzett[6] but the original statement has been substantiated by the work of Cross, Bevan and Heiberg,[7] who showed that not only phenol but catechol and hydroquinone were produced simultaneously when benzene was digested at low temperatures with peroxide of hydrogen and a trace of an iron salt. Hydroquinone has also been obtained by Gattermann and Friedrichs through the electrolytic oxidation of benzene in the presence of alcoholic sulphuric acid.[8] Although there can be no doubt of the power of peroxide of hydrogen to oxidize benzene, the reaction seems to have received no extended application to other compounds.

The only other reaction involving the introduction of hydroxyl groups into the benzene nucleus that will be referred to is an extremely interesting series of oxidations carried out with potassium persulphate, which are comprised in a number of Patent Specifications by Schering & Co. Thus the action of potassium persulphate in alkaline solutions upon phenols[9] or upon oxybenzoic acids results in the formation of peculiar sulphur-containing products which on treatment with acids yields dihydroxy derivatives. Salicylic acid, for example, yields hydroquinone carboxylic acid,[10] (2,5-dioxybenzoic acid) while para-oxybenzoic acid yields protocatechuic acid[11] (3,4-dioxybenzoic acid).

[1] Leeds: *Ber. d. deutsch. chem. Gesellsch.*, xiv, p. 975.
[2] Hoppe-Seyler's: *Zeitschr. f. physiol. Chem.*, p. 1552, 1879.
[3] Nencki and Sieber: *Journ. f. prakt. Chem.*, xxvi, p. 25.
[4] Radzisgewski: *Journ. f. prakt. Chem.*, xxiii, p. 96.
[5] Friedel and Crafts: *Compt. rend. de biol.*, lxxxiv, p. 1460, 1879.
[6] *Chem. News*, xliv, p. 229.
[7] *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2018.
[8] *Ibid.*, xxvii, p. 1942.
[9] E. Schering: D. R. P. 81068 (1894).
[10] *Ibid.*, 81297 (1894).
[11] *Ibid.*, 81298 (1894).

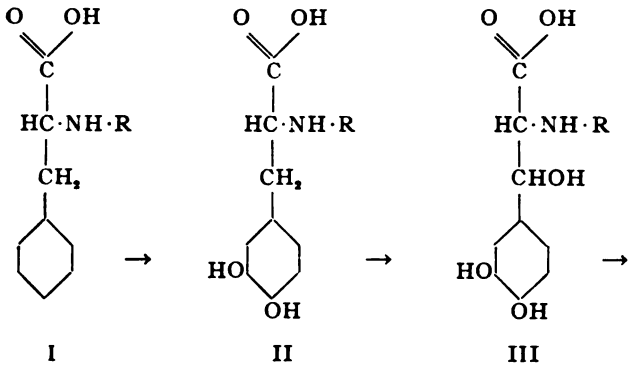
By employing potassium persulphate in sulphuric acid solution, for the oxidation of oxybenzoic acids, A. G. Perkin and his co-workers[1] have obtained substances resulting from the condensation of two molecules of the oxidation products of oxybenzoic acid. Thus para-oxybenzoic acid yields catellagic acid ($C_{14}H_6O_8$). Similar results are obtained by electrolytic oxidation of the oxybenzoic acids in sulphuric acid solution.

A considerable amount of evidence is gradually accumulating to emphasize the fact of the remarkably close similarity between oxidations effected by peroxide of hydrogen and those occurring in the organism, although it is not suggested that peroxide of hydrogen is the active agent in tissue oxidations but merely that the two types of reaction have much in common. It was therefore a matter of considerable interest to find that peroxide of hydrogen, acting at low temperatures, could effect the introduction of hydroxyl groups into the nucleus of aromatic acids, for this is a reaction which, though doubtless occurring in the animal organism, cannot be imitated by the more usual oxidizing agents.[2] Attempts to imitate in the laboratory reactions occurring in the organism are of value not only in confirming results already arrived at from a study of animal metabolism but also by affording grounds for speculation as to the mode of origin of other substances. For example, although no physiological evidence is at present available as to the mother substances of adrenalin,[3] yet the origin of this substance from tyrosin or phenylalanin can be easily pictured as taking place by a series of oxidations, entirely similar to those capable of being effected with peroxide of hydrogen. If the amido group of tyrosin or

[1] *Proc. Chem. Soc.*, xxi, p. 185; *Trans. Chem. Soc.*, lxxxix, p. 251.

[2] Attempts to oxidize ammonium benzoate, at low temperatures (50°) with sodium peroxide, magnesium peroxide, barium peroxide, and potassium persulphate and a mixture of potassium persulphate and silver oxide in no case gave results in any way comparable with those obtained with peroxide of hydrogen.

[3] Experiments which have been hitherto published upon the formation of adrenalin from tryptophan can hardly be considered convincing.

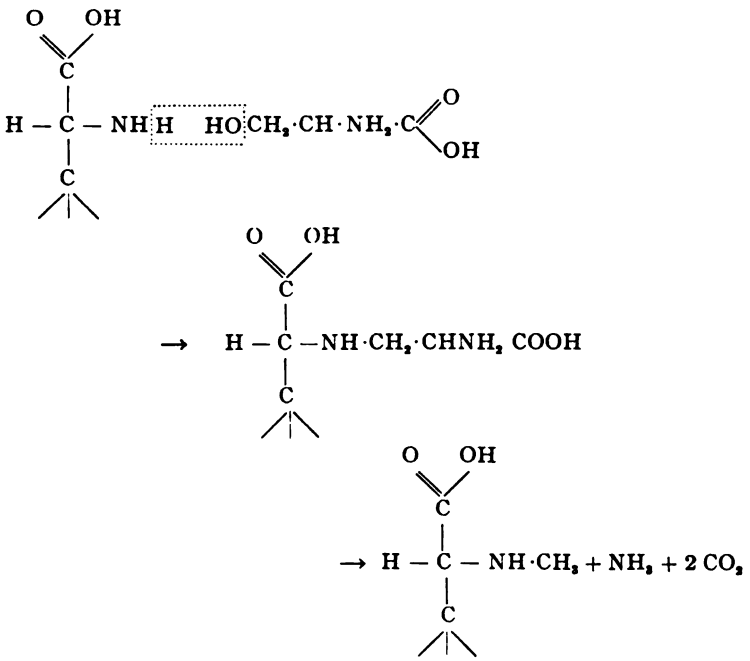


phenylalanin be assumed to be attached to some other carbon chain, such as an amido-acid group, as in the polypeptides, the tyrosin or phenylalanin grouping might well be protected from oxidation at the α carbon atom, while still being capable of undergoing nuclear and other oxidations. An example of this protection is seen in the fact that, although glycocoll is readily oxidized by hydrogen peroxide, yet if one of the hydrogen atoms of the amido group be substituted by an acid radical, *e. g.*, benzoyl, as in hippuric acid, the product is attacked with great difficulty.[1] Nuclear oxidation of the phenylalanin or tyrosin, with the introduction of two hydroxyl groups in positions (3) and (4) is easily intelligible from the similar formation of protocatechuic acid (3,4-dioxbenzoic acid) from benzoic acid, metaoxybenzoic acid and from paraoxybenzoic acid. Oxidation in the side chain, with introduction of an (OH) group in the β position (III) is a reaction with many parallels, for not only is β-oxidation common in the tissue oxidations of fatty acids, but actual examples of this have been observed in the oxidation of fatty acids, *e. g.*, butyric acid, with peroxide of hydrogen.[2] By removal of carbon dioxide the tyrosin derivatives would differ from adrenalin only as regards the nitrogen group. If the nitrogen were attached to the carboxyl group of the amido-acid radical, hydrolysis would result in the formation

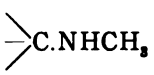
[1] Dakin: This *Journal*, i, p. 271, 1906.

[2] An account of these experiments will appear in the next number of this *Journal*.

of a product with an (NH_2) group instead of an $NHCH_3$ group, as is present in adrenalin. It is known, moreover, that the body can effect methylations, so that the substance might be converted into adrenalin in this way. If, however, the amido-group in the tyrosin molecule were originally combined with an oxyamido-acid grouping such as exists in serin



oxidative changes might well result in the formation of a



group. It is not impossible that the source of the methyl groups formed in the body is frequently due to such oxyamido-acid condensations. Many examples might be quoted of the analogy between peroxide oxidations and those occurring in the organism, but these will be reserved for future discussion when more experimental results have been obtained.

It is interesting at this point to consider briefly some possible modes of formation of phenolic substances in both the animal

and vegetable organism. In the latter, especially, they figure prominently as is seen from the extremely extensive list of phenols and phenolic-alcohols, -aldehydes, -ketones, -acids, tannins, etc., which are obtained from vegetable sources. There are two obvious processes by which phenolic substances may be produced, namely:

(1) Hydroxylation of preformed aromatic substances.

(2) Direct synthesis of phenols and their derivatives from aliphatic substances.

There is abundant proof that the first type of reaction commonly occurs in the animal organism, and several examples have already been quoted, *e. g.*, the production of phenol (Schultzen and Naunyn) and of catechol and hydroquinone from benzene,[1] the conversion of phenylalanin, tyrosin and oxyphenyllactic acid[2] into the homogentisic acid in alkaptonuria, the formation of para-amidophenol from anilin,[3] of acetylparamidophenol from acetanilide,[4] and many other examples.[5] But there is no evidence pointing to a synthesis in the animal body of phenols from aliphatic substances.[6]

[1] Baumann and Preusse: *Zeitschr. f. physiol. Chem.*, iii, p. 156; vi, p. 190.

[2] Neubauer and Falta: *ibid.*, xlii, p. 81, 1904.

[3] Fr. Müller: *Deutsch. med. Woch.*, 1887.

[4] Jaffe and Hilbert: *Zeitschr. f. physiol. Chem.*, xii, p. 295; K. Mörner, *ibid.*, xiii, p. 12.

[5] The following is a list of substances which in their passage through the animal organism undergo nuclear substitution by hydroxyl groups and are converted, at least in part, into mono- or poly-hydric phenols: Benzene, chlorobenzene, bromobenzene, *p*-dichlorobenzene, *m*-dichlorobenzene, *m*-cymene, isopropylbenzene, isobutylbenzene, methylethyltoluene, mesitylene, diphenyl, *p*-dibromdiphenyl, diphenylmethane, naphthalene, phenol, *o*-cresol, anisol, phenetol, guaiacol, thymol, phenylalanin, tyrosin, oxyphenylactic acid, anilin, formanilide, acetanilide, *o*-toluidin, *o*-acettoluidide, phenylurethane, carbonyl-*o*-amido-phenol, carbazol, acridin.

[6] A number of such syntheses have been accomplished outside the body but they are mostly of a kind such as could scarcely have any analogy in the living cell. Thus, hydroquinone and hydroquinone-dicarboxylic acid are formed by the action of potash upon succino-succinic ester, while small quantities of quinhydronedicarboxylic acid are formed by boiling free succino-succinic acid with water (Baeyer). Hydroquinone is also produced in the distillation of salts of succinic acid (Richter, *Journ. f. prakt. Chem.*, 2, xx, p. 207). Meta-oxyuvitic acid, $C_6H_2(CH_3)(OH)(COOH)_2$, is produced by the action of chloroform upon the sodium

In the plant cell, on the other hand, there is less evidence available, although Gonnemann's observation of the formation of homogentisic acid as an intermediate product in the action of tyrosinase upon tyrosin is an example of the first type of reaction, involving the hydroxylation of preformed benzene derivatives. Löw[1] and later Emmerling and Abderhalden[2] have observed the formation of protocatechuic acid by the action of fungi upon quinic acid (tetraoxyhexahydrobenzoic acid). Since the formation of quinic acid from carbohydrate is conceivable—although not yet demonstrated—it may be that this represents a direct synthesis of a phenol from aliphatic substances.

On the whole, it seems most probable that in both animal and plant life, phenolic substances are mainly, although possibly not exclusively produced by the hydroxylation of preformed benzene derivatives and not by direct synthesis from aliphatic substances. In other words, the introduction of hydroxyl groups into the nucleus of aromatic substances is a typical "biological" reaction.

EXPERIMENTAL PART.

Oxidation of Benzoic Acid. Benzoic acid (1 mol.) was suspended in a small quantity of warm water, and dissolved by addition of a slight excess of ammonia. Dilute neutral approximately 3 per cent hydrogen peroxide, the strength of which had been determined by titration with permanganate, was added in quantity equivalent to 1½ molecules of peroxide. Although the reaction proceeds at ordinary temperature, it is accelerated by heat, and accordingly the mixture was gently boiled

derivative of aceto-acetic ester (Oppenheim and Pfaff, *Ber. d. chem. Gesellsch.* vii, p. 929: Oppenheim and Precht, *ibid.*, ix, p. 321) while phloroglucintricarboxylic ester is obtained by heating malonic ester with sodium (Baeyer, *ibid.*, xviii, p. 3457) or with zinc alkyl derivatives (Lang, *ibid.*, xix, p. 2038). Collie and Myers (*Trans. Chem. Soc.*, lxiii, p. 124) have obtained orcin (= 3,5-dioxytoluene) by the action of alkali upon dehydracetic acid or dimethylpyrone while Berthelot (*Compt. rend. de l' Acad. des sci.*, cxxviii, p. 336) has shown that phenol is produced in small quantity by heating with potash the sulphonates resulting from the action of fuming sulphuric acid upon aldehyde or paraldehyde.

[1] *Ber. d. deutsch. chem. Gesellsch.*, xiv, p. 450.

[2] *Centralbl. f. Bakt.*, II, x, p. 338, 1903.

for several hours over a low flame with reflux condenser. A not inconsiderable amount of carbon dioxide is evolved during the boiling and since no free phenol appears to be formed, it is probable that part of the benzoic acid is completely oxidized. After heating the liquid was diluted, acidified with sulphuric acid and filtered. The precipitate consisted largely of unchanged benzoic acid but contained a considerable amount of salicylic acid. It was recrystallized several times from water till it reacted no more with ferric chloride. In this way about half the benzoic acid originally employed is recovered unchanged. The original filtrate and mother liquors are combined and repeatedly extracted with chloroform. The chloroform extract contained benzoic and salicylic acids. Meta- and para-oxybenzoic acids are insoluble in chloroform but are readily recovered by ether extraction.

The chloroform extract was evaporated and the residue gave the reactions for salicylic acid with ferric chloride, Millon's reagent, alkaline diazonium salts, etc. Part was dissolved in water and precipitated with bromine water. The precipitate was dried and recrystallized three times from chloroform. Tribromophenol-bromide, melting point 130–131°, was obtained in the form of thick prisms identical with the product similarly prepared from pure salicylic acid.

The ethereal extract containing the meta- and para-oxybenzoic acids was evaporated and extracted with a little hot chloroform to remove a trace of salicylic acid. The residue no longer reacted with ferric chloride. It was next dissolved in a little hot water and excess of barium hydroxide was added. The very sparingly soluble basic barium *p*-oxybenzoate crystallized out in the form of a sandy deposit. It was filtered off, decomposed with hydrochloric acid and the free acid recovered by ether extraction. It was recrystallized from water and formed large prisms, melting at 210–211°. The recorded melting points for *p*-oxybenzoic acid vary from 210–213°.[1] On treating the *p*-oxybenzoic acid in dilute aqueous solution with bromine water and repeatedly crystallizing the dried precipitate from chloro-

[1] Hartmann: *Journ. f. prakt. Chem.*, 2, xvi, p. 36; Negri: *Gaz. chem. ital.*, xxvi, I, p. 65.

form, tribromphenolbromide, melting point 130–131°,[1] was readily obtained. The para-oxybenzoic acid showed the usual reactions with Millon's reagent, diazonium salts. It gave no coloration with ferric chloride.

The filtrate from the basic barium *p*-oxybenzoate was acidified and extracted with ether. The extract was evaporated and crystallized from water. Since the preparation of pure meta-oxybenzoic acid possessing the correct melting point presented some difficulties, as some of the para-oxybenzoic acid was still present, it was converted into its bromine derivative. The residue was dissolved in water and excess of bromine water added; the precipitate of tribromphenol bromide, derived from the *p*-oxybenzoic acid was filtered off and the filtrate extracted with ether. The ethereal extract was dissolved in hot water, treated with a little charcoal and on cooling 2,4,6-tribrom-3-oxybenzoic acid, melting point 145–146°, was readily crystallized out in the form of tufts of needles. Herzig[2] gives 145–147° for the melting point of tribrom-meta-oxybenzoic acid. The product obtained was identical in appearance with that prepared from pure meta-oxybenzoic acid.

The total yield of the three oxybenzoic acids was determined by iodine titration and amounts to 15 to 20 per cent of theory. The amount of each acid which can be separated in the pure state is much smaller, however.

Oxidation of Para-oxybenzoic Acid. One molecular proportion of *p*-oxybenzoic acid was dissolved in hot water and ammonia added until about five-sixths of the acid had been neutralized. Three per cent hydrogen peroxide (1.5 g. mol.) was then added and the mixture digested on the water bath for some hours. A considerable amount of carbon dioxide is given off so that probably a portion of the oxybenzoic acid is completely oxidized. If the reaction of the fluid is allowed to be decidedly alkaline considerable darkening results on oxidation. After oxidation the liquid was acidified and the products of oxidation, along with much unchanged *p*-oxybenzoic acid, were extracted with ether.

[1] Auwers and Büttner: *Ann. d. Chem.*, 302, p. 133, give 131° for the melting-point of tribromophenolbromide. Previously the melting-point had erroneously been recorded as 118°.

[2] *Monatsh. f. Chem.*, xix, p. 92.

The residue was tested for 2,4-dioxybenzoic acid and 3,4-dioxybenzoic acid (protocatechuic acid), these being the only dioxybenzoic acids derivable from *p*-oxybenzoic acid. The former acid was found to be absent for no reaction was obtained with bleaching powder, and moreover the dioxy-acid formed was precipitable by lead acetate, whereas 2,4-dioxybenzoic acid is not precipitated by this reagent. The residue was then dissolved in hot water and on cooling a large amount of unchanged *p*-oxybenzoic acid crystallized out.[1] The filtrate was then precipitated with basic lead acetate. The precipitate on decomposition with sulphuretted hydrogen and concentration of the aqueous solution yielded crystals of protocatechuic acid. After recrystallizing from water the acid melted at 199–200°. The product gave an intense blue-green coloration with ferric solution, which turned dark red on addition of caustic soda solution. It reduced ammoniacal silver and on fusion with potash it yielded catechol. The yield of dioxybenzoic acid was not more than 5 per cent of the theoretical amount.

Oxidation of Meta-oxybenzoic Acid. The reaction was carried out, and the products of oxidation separated in exactly the same way as that employed for the para-oxybenzoic acid. Of the four dioxybenzoic acids which might theoretically be formed only the 3,4-acids (protocatechuic acid) could be identified. It was separated and purified as in the preceding case. The yield as before was small. The acid gave all the reactions of protocatechuic acid. 2,5-Dioxybenzoic acid was tested for, with negative result, by means of the quinone reaction with ferric chloride.[2] 3,5-Dioxybenzoic acid, which gives no color reaction with ferric chloride, could not be detected with the anthrachyrson reaction for this acid. 2,3-Dioxybenzoic acid was excluded by reason of the fact that the oxidation products gave no blue color with ferric chloride, turning violet-red on addition of alkali.

Oxidation of Salicylic Acid. The oxidation was carried out as with meta- and para-oxybenzoic acids. The liquid, which had turned dark brown was acidified and much unchanged salicylic

[1] By again oxidizing this precipitate of unchanged para-oxybenzoic acid, the yield of the dioxybenzoic acid may be much increased, especially if the oxidation of the unchanged acid be repeated several times.

[2] Nef: *Ber. d. deutsch. chem. Gesellsch*, xviii, p. 3499.

acid filtered off. The filtrate extracted repeatedly with ether. The dry ethereal extract was then treated with warm chloroform to remove unchanged salicylic acid. The insoluble residue was small and somewhat discolored. It gave a strong blue-black coloration with ferric chloride which turned violet-red on addition of sodium bicarbonate and was completely precipitable by lead acetate. Since 2,4-, 2,5-, and 2,6-dioxybenzoic acids are not precipitable by lead acetate, these acids could not have been present in appreciable amounts. The reactions with ferric chloride and with lead acetate agree with those of 2,3-dioxybenzoic acid. The acid was purified by means of its lead salt. By decomposing the lead salt with sulphuretted hydrogen, the free acid was obtained in the form of colorless needles, m. p. 200–202°. It strongly reduced ammoniacal silver solution, even in the cold and also Fehling's solution on boiling. It gave catechol on dry distillation. The yield of acid was about 3 per cent.

Oxidation of Substitution Derivatives of Benzoic Acid. Several substituted benzoic acids were oxidized by the same method that was employed for the oxidation of benzoic acid. After heating the solutions were acidified, extracted with ether and tested for phenolic (OH) groups by ferric chloride, Millon's reagent and with sodium diazobenzenesulphonate in the presence of excess of sodium carbonate. No attempt was made to isolate the individual products. The yields of hydroxy-acids in all cases appears to be very small.

Acids Oxidized.	Reaction of Products of Oxidation with the Following Reagents.		
	Ferric Chloride.	Millon's Reagent.	Diazo Reaction.
Orthochlorobenzoic acid	Violet color	Strong red	Orange-red.
Parabromobenzoic acid	Violet-red color	Very faint positive reaction.	Orange.
Paranitrobenzoic acid	Violet-red color.	Positive but faint.	Orange-red.
Metadinotrobenzoic acid (1,3,5.)	Negative	Negative	Strong orange red.*
Ortho-amido-benzoic acid.†	Violet-red	Positive	Orange-red much deeper than the amido benzoic acid.

* The original acid gave no trace of color reaction with diazonium salts.
† The usual addition of ammonia was not made in the oxidation of this acid.

It will be seen from the table that qualitative evidence of the formation of phenolic acids was obtained in each case. It is

possible that the oxidation of the amido-acid with peroxide yielded a phenolic acid through replacement of the amido group as is known to occur with some oxidizing agents, but no similar replacement would be likely to occur with the other benzoic acids.

SUMMARY.

I. Hydrogen peroxide acting upon the ammonium salts of benzoic acid, or its chlorine, bromine, nitro- and amido-derivatives, can introduce hydroxyl groups into the nucleus, although the yield of phenolic acid is small. Hippuric acid undergoes nuclear oxidation with difficulty.

II. In the case of benzoic acid itself, ortho-, meta- and para-oxybenzoic acids are produced in not widely differing amounts. On further oxidation both meta- and para-oxybenzoic acids give protocatechuic acid (3,4-dioxybenzoic acid). Salicylic acid gives 2,3-dioxybenzoic acid. The second hydroxyl group in the dioxybenzoic acids takes up a position ortho to that already in the ring. A part of the benzoic acid is decomposed with formation of carbon dioxide and possibly other products.

III. The reaction occurs in approximately neutral solution and to some extent at least the reaction progresses at the ordinary temperature. Although benzoic acid itself is not appreciably oxidized in the body, possibly owing to its conversion into hippuric acid, a close parallelism exists between oxidations carried out under these conditions and those taking place in both vegetable and animal tissue oxidations.

IV. A summary is given of the reactions which have hitherto been successful in effecting the direct entrance of hydroxyl groups into the nucleus of benzene derivatives outside the body, together with a list of aromatic substances which undergo nuclear hydroxylation in their passage through the animal body.

V. The possible origin of phenolic substances in vegetable and animal tissues is considered and though there is ample proof of the production of phenolic substances by the oxidation of preformed aromatic substances, the conclusion is reached that as far as the limited experimental results permit there is little evidence that they originate directly from the condensation or rearrangement of aliphatic substances.

THE ACTION OF ARGINASE UPON CREATIN AND OTHER GUANIDIN DERIVATIVES.

By H. D. DAKIN.

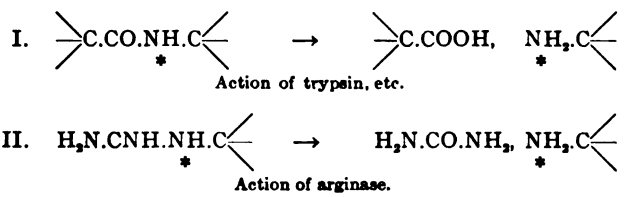
(*From the Laboratory of Dr. C. A. Herter, New York.*)

(Received for publication, September 1, 1907.)

Three years ago Kossel and Dakin[1] described the action of an enzyme which had the property of effecting the hydrolysis of arginin, with production of urea and ornithin.

$$H_2N - \overset{\overset{\displaystyle NH}{\|}}{C} - NH.CH_2.CH_2.CH_2.CH(NH_2).COOH + H_2O = H_2N.CO.NH_2 + H_2N.CH_2.CH_2.CH_2.CH(NH_2)COOH$$

This reaction represented a new type of enzymic hydrolysis, differing from that effected by other "related" enzymes, such as pepsin, trypsin and erepsin, in the fact that the latter only effect separation of amido-acid groupings at points where an imide group is adjacent to a carbonyl group (I), while in the case of arginase a C:NH group may occupy the place of the carbonyl grouping (II):



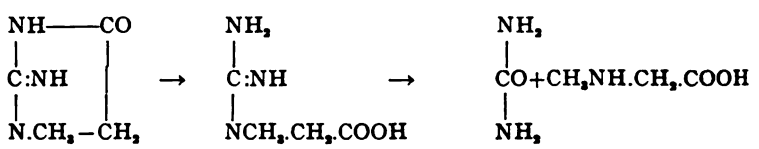
This enzyme named arginase was found to be most abundant in the liver, but was present in the kidney, intestinal mucous membrane, thymus and probably other organs. It was also found by Shiga[2] among the enzymes obtained from yeast. In addition to arginin, arginase acts upon the arginin complex

[1] *Zeitschr. f. physiol. Chem.*, xli, p. 321, 1904; xlii, p. 131, 1904; *Münch. med. Wochenschr.*, 1904, No. 13.

[2] *Zeitschr. f. physiol. Chem.*, xlii, p. 502, 1904.

present in certain protones, with the liberation of urea and production of polypeptide-like substances which yield ornithin on complete hydrolysis with acids. Arginase was found to be without action on the protamines themselves as well as other proteins.

The fact that arginase attacked the guanidin complex in arginin at once suggested that the same enzyme might possibly attack other guanidin derivatives. It was found, however, that arginase had but little action on guanidin itself and negative results were also obtained in a preliminary experiment with creatinin, but the great interest that attaches to creatin and creatinin metabolism made it desirable to determine more exactly whether creatin and creatinin were attacked at all by arginase. While these experiments were in progress a paper appeared by Gottlieb and Stangassinger[1] in which enzymes are described which act upon creatin and creatinin, respectively. These enzymes are named creatinase and creatininase. The former apparently converts creatin into creatinin, while the latter decomposes creatinin. The products of decomposition of creatinin are not described, but if the reaction is similar to the action of arginase (and Gottlieb and Stangassinger state that a long preserved arginase preparation exerted an appreciable action upon creatinin) the products should be sarcosin (methyl glycocoll) and urea. If this should be the case, it would appear anomalous that the first step in the enzymic decomposition of creatin should consist in its conversion into its anhydride—creatinin—for the hydrolysis of the latter with formation of sarcosin and urea would apparently necessitate the re-formation of creatin as an intermediate product.



The object of the following note is to place on record the results of some experiments which indicate that arginase and creatininase are different enzymes, for organ extracts prepared

[1] *Zeitschr. f. physiol. Chem.*, lii, p. 1, 1907.

in a manner such as would furnish an extremely active arginase preparation were found to be practically without action upon both creatin and creatinin as well as some other guanidin derivatives. The organs examined were the liver, kidney and duodenal mucous membrane of the calf and dog. It is remarkable that the decomposition of creatin and creatinin described by Gottlieb and Stangassinger was not observed in these extracts and the cause of this failure is by no means clear. It is hoped, however, that further experiments will throw light on this discrepancy. It is interesting to note that the changes induced by Gottlieb's enzyme are extremely slow in comparison with the vigorous action of arginase, for while the former requires many hours or days to effect considerable decomposition, a small quantity of the latter is able to decompose several grams of arginin in a few minutes.

In the following experiments the tissues were ground up and shaken vigorously with five to ten times their weight of water. with liberal addition of toluene. The fluids were then roughly strained through gauze and definite volumes of the turbid extract were employed. Similar results were obtained when more highly purified enzyme preparations were employed, obtained by alcohol and ether precipitation in the manner adopted for the preparation of arginase. Additions of creatin or creatinin were made to these extracts, which were then digested at 37° for varying lengths of time. The creatinin was estimated by coagulating measured portions of the digested fluid with dilute acetic acid at the boiling point, filtering, and then applying Folin's colorimetric method with picric acid and caustic soda. Creatin was estimated in a similar way, after conversion into creatinin. The latter change was effected by boiling the clear filtrate with normal hydrochloric acid for two hours, evaporating on the water-bath and determining the total creatin plus creatinin. The difference in preformed creatinin and the creatinin after treatment with acid was a measure of the creatin. The amount of solution employed for colorimetric estimation was such that its creatinin content varied from eight to twelve milligrams. Appropriate blank experiments were carried out and the necessary corrections applied.

Dog liver extract upon creatinin.

One hundred cc. of 10 per cent emulsion used.

Creatinin added	= 0.0794 gm
" found after 72 hours	= 0.0797 "
" " " 240 "	= 0.0792 "

Dog liver extract upon creatin.

One hundred cc. of 10 per cent emulsion used.

Creatin added	= 0.0604 gm.
" found after 72 hours	= 0.0569 "
" " " 240 "	= 0.0582 "

Dog kidney extract upon creatinin.

One hundred cc. of 10 per cent emulsion used.

Creatinin added	= 0.0796 gm.
" found after 72 hours	= 0.0806 "
" " " 240 "	= 0.0860 "

Dog kidney extract upon creatin.

One hundred cc. of 10 per cent emulsion used.

Creatin added	= 0.0604 gm.
" found after 72 hours	= 0.0660 "
" " " 240 "	= 0.0594 "

Calf liver extract upon creatinin

One hundred and twenty cc. of 20 per cent emulsion used.

Creatinin added	= 0.115 gm.
" found after 160 hours	= 0.120 "

Duodenal mucous membrane from calf upon creatinin.

One hundred cc. of 12 per cent emulsion used.

Creatinin added	= 0.123 gm.
" found after 40 hours	= 0.134 "
" " " 188 "	= 0.112 "

All these extracts were prepared in a manner such as yields extremely active arginase preparations, yet the results shown above do not indicate that they had any perceptible action upon creatin and creatinin. The small differences between the amounts of creatin and creatinin added and those found after digestion are mostly well within the limits of experimental error and it is therefore concluded that in all probability arginase has nothing in common with Gottlieb and Stangassinger's creatinase and creatininase.

Assuming therefore that arginase does not act upon creatin or creatinin, it is interesting to inquire into the reason why such similarly constituted guanidin derivatives are not attacked by the same enzymes. The most obvious difference in the con-

stitution of arginin and creatin, judged from the point of view of their attack by urea-forming enzymes, is the fact that while the former contains a guanidin complex, the molecule of the latter contains a methylguanidin group. It is improbable, however, that this constitutes the reason for the differing behavior towards arginase, for in the first place guanidin itself is not appreciably attacked by arginase, as was found by Shiga for the enzymes from yeast and by the writer for liver arginase.[1] The result is in agreement also with the fact that guanidin injected into the animal organism is almost entirely excreted unchanged in the urine. The recognition by Kutscher, of methylguanidin as a urinary constituent, makes it probable that arginase has also no action upon methylguanidin, although direct experiments upon this point are still wanting. Incidentally it may be noted that liver extracts are also without action upon triphenylguanidin.

It might be urged that the explanation of these negative results with guanidin and its alkyl derivatives is to be found in the absence of an aliphatic chain of carbon atoms containing a carboxyl group, such as is present in the arginin molecule; but this again is improbable, for it was found that arginase was without material action upon glycocyamin (guanido-acetic acid). This substance was prepared by Nencki and Sieber's method[2] by heating guanidin carbonate with glycocoll. It was digested with tissue extracts containing arginase as in the other experiments and the digested fluids were examined for increased urea-formation.[3]

$$NH_2-\overset{\overset{\displaystyle NH}{\|}}{C}-NH.CH_2.COOH+H_2O=NH_2.CO.NH_2+NH_2.CH_2.COOH$$

[1] Guanidin carbonate was digested with calf liver extracts. The digested fluids were subsequently examined for increased ammonia and urea production, with negative results.

[2] *Journ. f. prakt. Chem.*, 2, xvii p. 477.

[3] Urea was estimated by removing the coagulable protein from the liquids, concentrating at low temperatures and then treating with ether and alcohol, etc., as in the Mörner-Sjöqvist method. The urea was subsequently precipitated with mercuric nitrate and sodium carbonate and finally estimated by Kjeldahl nitrogen determination.

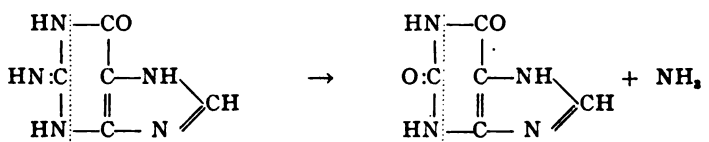
The negative results with glycocyamin show that the absence of action of arginase upon creatin is *not* due to the presence of a methyl group in the latter and also that the reason of its failure to decompose guanidin itself and its alkyl derivatives is not to be sought in the absence of an aliphatic chain containing a carboxyl group.[1]

It is probable that arginase is essentially a specific enzyme and that, as in the case of the sugars and glucosides, a very close inter-relation of enzyme and substrate is necessary for hydrolysis to occur. It must be remembered that arginin is an optically active substance and although the asymmetric carbon atom is situated at a considerable distance from the guanidin complex which is attacked by the arginase,

$$H_2N.C.NH.NH.CH_2.CH_2.CH_2.\overset{*}{C}HNH_2.COOH$$

yet it undoubtedly exerts a determining influence upon the course of the reaction. The proof of this lies in the fact that, as Otto Riesser[2] has shown, if racemic arginin be digested with arginase, the dextro-compound is hydrolyzed, whilst lævo-arginin remains unattacked. Regarded simply from the standpoint of structure, excluding the stereo-chemical relationship, there is no apparent reason why lævo-arginin should not undergo hydrolysis with arginase in the same manner as dextro-arginin even though the rates of hydrolysis should be dissimilar, as is the case with the hydrolysis of isomeric optically active esters by lipase.[3]

[1] It may not be out of place to draw attention to the close similarity existing between arginase and guanase both in their distribution in the body and also the fact that both attach substances containing the guanidin grouping with formation of a urea-group and either ammonia or an amido acid.



[2] *Zeitschr. f. physiol. Chem.*, xlix, p. 210, 1906.

[3] Dakin: *Proc. Chem. Soc.*, xix, p. 161, 1903; *Journ. of Physiol.*, xxx, p. 253, 1903; xxxii, p. 199, 1905.

The evidence at present available, therefore, supports the belief that arginase is a specific enzyme adapted for the exclusive hydrolysis, as far as is known, of dextro-arginin or of substances containing the dextro-arginin grouping and that, as in the case of the glucosides and sugars, the relation of the enzyme to the substrate is of so intimate and finely adjusted a kind, that many other substances structurally similar to arginin are incapable of hydrolysis by arginase.

ON THE CHEMISTRY OF BACILLUS COLI COMMUNIS.

II. THE NON-POISONOUS PORTION.

By MARY F. LEACH.

(*From the Hygienic Laboratory of the University of Michigan, Ann Arbor.*)

(Received for publication, July 22, 1907.)

Introduction. In previous papers upon this subject[1] the author has described the method in use in this laboratory for obtaining large amounts of bacterial cellular substance, and has shown that the cell substance of *Bacillus coli communis*, after thorough extraction with alcohol and ether, is wholly dissolved by the successive action of dilute acid and alkali. The solutions thus obtained show the presence of proteid, nucleo-compounds, and carbohydrate, while the properties and reactions suggest that the cell is largely composed of glyco-nucleo-proteid. Heating with 1 per cent sulfuric acid breaks off the carbohydrates, and splits up the proteid, apparently causing cleavage along definite lines. By digestion with stronger acid, xanthin and hexon bases were obtained. Lysin was isolated as the picrate, purified, and transformed into the chlorid, both of which were proved to be identical with lysin picrate and chlorid from other sources. Thus bacterial proteid, like all others thus far examined, contains the hexon group, and another point of resemblance is established to other proteids of both animal and vegetable origin.

Additional work shows[2] that the cellular substance of the colon bacillus may be separated into a poisonous and a non-poisonous portion by the action of a dilute alcoholic solution of sodium hydrate. By the same method other proteids have

[1] *Trans. of Assoc. of Amer. Phys.*, xvii, p. 274, 1902; *Journ. of the Amer. Med. Assoc.*, xlii, p. 1003, 1904; this *Journal*, i, p. 463, 1906.

[2] Wheeler: "The Extraction of the Intracellular Toxin of the Colon Bacillus," *Journ. of the Amer. Med. Assoc.*, xliv, p. 1271, 1905.

been split up into poisonous and non-poisonous portions.[1] By means of the non-poisonous portion of the colon bacillus, it is possible to induce active immunity to virulent cultures of the living germ.[2] Moreover the non-poisonous portion of the typhoid bacillus has been used with marked success in the treatment of typhoid fever. Hence the chemical nature of the non-poisonous part of the bacterial cell is of great interest and importance. Further a single injection of many proteids, both of vegetable and of animal origin, so sensitizes the animal, that a second treatment, after a certain time limit has passed, will prove promptly fatal. The non-poisonous part of any proteids that have been thus far examined, will sensitize toward the whole proteid, but is entirely specific in its action.[3] It is the purpose of this paper to give an account of the non-poisonous part of the colon bacillus, and of some of its decomposition products, especially the relation of the nitrogen and phosphorus.

MATERIAL. *Bacillus coli communis* was raised upon agar in large tanks, harvested and purified as described elsewhere. By repeated boiling with sodium hydrate dissolved in absolute alcohol,[4] about one-third of the cellular substance, including all the poison, goes into solution. The insoluble residue is filtered out, extracted in Soxhlets with alcohol for 20 to 30 hours, dried and pulverized. This is the material used in the following investigation.

PROPERTIES. The non-poisonous portion of cell substance remaining after repeated extraction with alcoholic soda as described above, is a cream colored powder. On burning it puffs up, gives off the odor characteristic of nitrogenous compounds, and leaves a copious ash containing phosphate. The substance is mainly soluble in water, giving an opalescent solution from which a light colored sediment settles out on standing, leaving a clear golden brown solution. This sediment is not

[1] Vaughan and Wheeler: "Experimental Immunity to Colon and Typhoid Bacilli," *N. Y. Med. Journ.*, lxxxv, p. 1170, 1907.

[2] V. C. Vaughan, Jr.: "The Production of Active Immunity with the Split Products of the Colon Bacillus," *Journ. of Med. Res.*, xiv, p. 67, 1905.

[3] Vaughan and Wheeler: "Effects of Egg-White on Animals;" *Journ. of Infect. Dis.*, iv, p. 476, 1907.

[4] Wheeler: *loc. cit.*

dissolved by dilute hydrochloric acid or dilute sodium hydrate in the cold, but is dissolved by boiling with alkali. The clear aqueous solution is alkaline in reaction from sodium hydrate either held mechanically, or combined chemically; it is precipitated by mineral acids and by alcohol. It gives the biuret, xanthoproteic, and Adamkiewicz tests; Millon's test is not very satisfactory, but is more pronounced if the alkali is first neutralized. It does not reduce Fehling's solution directly, but does so after boiling with hydrochloric acid. Tests with α-naphthol, phloroglucin and orcin give positive results. Ammonium molybdate gives an organic precipitate, but no evidence of free phosphate. Thus the preliminary tests show the presence of proteid, nucleic and carbohydrate groups. Comparing these results with work previously reported upon the portion of the cell which is soluble in alkaline alcohol, the soluble part always gives a very pronounced Millon test, contains no carbohydrate, and all the toxophorous group; while the portion insoluble in alkaline alcohol gives only a faint Millon test, contains all the carbohydrates, most of the phosphorus, and seemingly the haptophorous group. (Haptophorous is used here in Ehrlich's sense of readily combining with the physiological constituents of living cells.)

Cleavage Products. With bacterial cell substance we have succeeded in establishing certain definite lines of cleavage; various attempts were made to find lines of cleavage for the non-poisonous portion. The most promising ones may be outlined as follows:

I. The material was treated with acid alcohol giving a solution A and an insoluble residue B. A solution of B in aqueous alkali, on addition of alcohol, gave Filtrate C and Precipitate D.

II. An aqueous solution of the material treated with dilute acetic acid gave Precipitate K and Filtrate L. On treating L with alcohol, Precipitate M and Filtrate N were obtained.

III. Again an aqueous solution of material gave with acid alcohol, Precipitate G and Filtrate H.

As was to be expected, qualitative tests showed few marked differences in these preparations, but quantitative differences were found which will be discussed later.

Alcoholic Solutions. As the cell substance of the colon germ is entirely dissolved by successive treatment with very dilute

aqueous acid and alkali, the portion insoluble in alcoholic soda was treated with acid alcohol. Doubtless the prolonged boiling in extracting the poison has pronounced effect upon the physiological constituents of the cell, still the substance carrying immunity is to some extent left intact. As so many such substances are sensitive to heat, the further extractions were carried on at room temperature.

Fifty grams of haptophorous substance were mixed with one liter of absolute alcohol to which 30 cc. of strong hydrochloric acid had been added, and shaken in a mechanical shaker for 5 hours at room temperature. After standing over night it was filtered. The residue, which will be designated as B, was washed with alcohol until the washings were no longer acid to moist litmus, dried and pulverized. The weight was approximately 45 grams. The filtrate was evaporated to dryness on a water bath. A dark, sticky mass resulted, which will be designated A. When dry it could be pulverized, but was very hygroscopic. After pulverizing it weighed 7 grams. A is mainly soluble in water. With the aqueous solution the xanthoproteic, α-naphthol, phloroglucin, and orcin tests are all positive, the Millon test is faint, biuret negative. It burns with a suggestion of burning feathers, leaving an ash with a greenish tinge. Tests showed the presence of aluminum and of phosphate in the ash. Thus qualitative tests show that acid alcohol dissolved out some carbohydrate, inorganic matter, as well as nucleo- and proteid decomposition products, but no undecomposed proteid, while about nine-tenths of the substance was left intact. Ash, nitrogen and phosphorus were determined as described later.

On stirring with water, B forms an emulsion which is acid in reaction. The addition of sodium bicarbonate to slight alkalinity gives a clear, dark solution which responds to the biuret, xanthoproteic and Millon tests. The biuret is reddish violet. Attempts were made to precipitate the proteid from this alkaline solution. Hydrochloric acid gave a suspension which seemed to thicken on heating, did not settle, and passed through filter paper. Ammonium sulfate solution gave no precipitate, but on adding the dry salt to saturation, precipitation took place, the precipitate settled well and filtered clear. Both aqueous and alcoholic solutions of mercuric chlorid with a little acid gave

suspensions which did not settle. Alcoholic solution of picric acid, copper sulfate and alcohol, and alcohol alone, all gave precipitates that settled well. As alcohol seemed most promising, a clear alkaline solution of B was acidified with hydrochloric acid, and poured slowly with constant stirring into 5 volumes, giving a voluminous, flocculent white precipitate, D. This was filtered out and washed with alcohol. It was readily soluble in water, gave Millon, xanthoproteic, and α-naphthol tests. The ash contained phosphate.

The alcoholic filtrate from D was evaporated to dryness, and the residue extracted repeatedly with alcohol. The residue consisted mainly of sodium chloride. The extracts were evaporated to dryness leaving a small amount of brown scale, C, upon the dish. C burns with nitrogenous odor, leaving comparatively little ash which is largely phosphate. The solution gave xanthoproteic and Millon tests. Owing to lack of material no quantitative determinations were made. As A, B, C and D showed no very marked differences, other methods of cleavage were tried.

Aqueous Solutions. In preparing aqueous solutions it was found best to add water to the material little at a time, with careful stirring, first making a smooth stiff dough, then gradually thinning it to the proper consistency. The mixture was then shaken at room temperature for two or three hours.

Ten grams of air-dried material mixed with 300 cc. of water was filtered through a sterilized Pasteur filter, requiring 36 hours. The insoluble portion was removed from the filter, using proper precautions to avoid removing particles of the filter. It was mixed with a large amount of water and filtered through both hard and soft paper, with and without using suction. No device was successful in getting the residue reasonably free from solution. By repeated treatment nearly all went into an opalescent solution from which nothing separated out on standing. The remainder when dry looked like agar and sand.

Aliquot portions of the above solution that had been filtered through porcelain were used for determinations of nitrogen, phosphorus, diamino- and monamino-nitrogen, according to methods to be described later. Ten cc. contained 0.0036 gram of phosphorus and 0.008092 gram of nitrogen, corresponding to 0.090 gram of phosphorus and 0.2023 gram of nitrogen in the

whole. As the non-poisonous portion of the germ contains 5.556 per cent of nitrogen and 2.34 per cent of phosphorus, less than half passed through the filter. The ratio of nitrogen to phosphorus is 2.25, very close to the ratio in the original, and in a number of the preparations included in a subsequent table. Of the total nitrogen in the solution, 15.91 per cent was in the form of diamino compounds, and 73.06 per cent as monamino compounds.

As filtering through porcelain kept back most of the substance, that was abandoned. A 5 per cent solution was filtered through cotton with suction, leaving very little upon the filter. The filtrate was not quite clear, so it was filtered twice through a "Nutsche" with two soft filters, then twice with hard filter without much effect. However as opalescence seemed to be due to matter in solution rather than suspension, further samples were filtered through a layer of cotton and then through soft filters with suction.

The difficulty of filtration, the mucilaginous character of the solutions, as well as other properties and reactions, suggested mucin, while the presence of nucleic acid was also indicated. In the hope of effecting a separation, dilute acetic acid was added to an aqueous solution prepared as above, giving a brown precipitate, K, and a filtrate, L. Precipitate K was washed with alcohol and then with ether, and dried *in vacuo*. The yield was 0.6 gram from 20 grams of substance. It gave xanthoproteic tests but not the biuret, and contained phosphorus, hence it was not mucin.

Again 50 grams of material was dissolved and acetic acid was added to the solution, giving Precipitate K_2 and Filtrate L_2. The yield of K_2 was 8 grams. It does not give a clear solution with water, but clears on the addition of a very little sodium acid carbonate. K gives the xanthoproteic and α-naphthol tests, a faint Millon, no biuret. Ammonium molybdate solution gives no evidence of phosphate, but there is phosphorus present in organic combination. Many indications suggest nucleic acid, while the precipitation with acetic acid points toward guanylic acid.[1]

[1] Bang: "Chemische und physiologische Studien uber die Guanylsäure," *Zeitschr. f. physiol. Chem.*, xxxi, p. 411, 1900–1.

Filtrate L was poured into two volumes of alcohol, giving Precipitate M and Filtrate N. The precipitate was washed with alcohol and ether. After drying *in vacuo* and powdering there was 3½ grams of a fine mealy powder almost white, readily soluble in water. It turned yellow on heating with sodium hydrate, the xanthoproteic test was positive, the biuret negative.

Filtrate L_2 was treated in the same way. The precipitate, M_2, was sticky at first, but the sticky substance was removed by repeated treatment with alcohol and ether. The yield of M_2 was 13 grams. It is not so soluble in water as M, but the solution clears on neutralization with sodium bicarbonate. Animal experiments and determinations of nitrogen, phosphorus and ash did not show sufficient difference between K and M to warrant further use of acetic acid.

Again a 5 per cent aqueous solution of the immunizing portion of the germ was acidified and tested with varying amounts of 50 per cent alcohol, absolute alcohol, also alcohol and ether. As the result of these tests, the solution was poured into 4 volumes of absolute alcohol containing 10 cc. of hydrochloric acid and 100 cc. of ether per liter. After settling, the supernatant liquid was siphoned off, the Precipitate G filtered with suction, washed with alcohol containing ether, then with ether, dried and pulverized. The yield from 50 grams of material was 19 grams. This precipitate was twice dissolved in water made faintly alkaline with sodium acid carbonate, and reprecipitated by alcohol containing hydrochloric acid and ether. The final precipitate G weighed 16 grams. With water G forms an emulsion acid in reaction, cleared by the addition of alkali. The biuret test is negative, Millon doubtful, xanthoproteic, Adamkiewicz, α-naphthol, and orcin tests are all positive, the carbohydrate tests being very marked. After boiling with acid there is copious reduction of Fehling's solution. The substance was tested for glycogen with negative results.

The alcoholic filtrate from G was concentrated on a water bath below the boiling point. A black, charred mass H was left, easily pulverized, smelling strongly of hydrochloric acid. It contained phosphate, and responded to xanthoproteic, Millon, and α-naphthol tests, but did not reduce Fehling's solution even after boiling with acid.

Ash, Nitrogen and Phosphorus. On heating, these substances puff up, forming liquid and volatile products, some of which burn with a flame. For determinations of ash a sample was heated in a platinum crucible to low redness, using extreme care to completely incinerate the organic matter, without volatilizing the inorganic portion. The results are tabulated under the heading "Ash." All determinations were made in duplicate, and figures given are the average of closely agreeing determinations. In some cases the ash obtained as above was heated with the full flame of a Detroit burner, and results are given under the heading "Fixed Ash." Solutions of ash gave immediate precipitates in the cold with ammonium molybdate, and hence contain orthophosphate. If the phosphorus in the ash is all combined as PO_4, then

$$\text{Wt. } P : \text{Wt. } PO_4 :: 31 : 95$$

or,

$$PO_4 = P \times 3.06$$

Now the phosphorus in the material is organic, and the PO_4 should not be counted as mineral matter. The total ash less $P \times 3.06$ is given in the table under the heading "Inorganic Ash," and these figures are the ones used in calculating ash-free N and P.

Nitrogen determinations were made by the Kjeldahl-Gunning method. Phosphorus was determined by the Neumann[1] method with some modifications. The sample was shaken with ammonium nitrate in a 500 cc. Kjeldahl digestion flask,[2] and 10 cc. sulfuric acid added. When frothing subsided it was heated carefully on a sand bath, more ammonium nitrate added, and heated until colorless. The mixture was cooled, water added, more ammonium nitrate, made alkaline with ammonia, then acid with nitric acid adding 1 cc. in excess. It was then heated until bubbles rose. At the same time ammonium molybdate solution was heated until bubbles rose, and poured slowly into the hot phosphate solution with shaking or stirring. Prepared thus the

[1] "Einfache Veraschungsmethode," *Zeitschr .f. physiol. Chem.* xxxvii, p. 115, 1902–3; xliii, p. 32, 1904–5.

[2] Sherman: "Determination of Sulfur and Phosphorus in Organic Materials," *Journ. of the Amer. Chem. Soc.*, xxiv, p. 1100, 1902.

precipitate settled within 15 minutes,[1] although it was always left for an hour or more. The precipitate was washed with 0.1 per cent ammonium nitrate solution[2] until free from acid, filter and precipitate returned to the flask, 150 cc. of water added, half-normal sodium hydrate run in from a burette until the precipitate is dissolved, 5 or 6 cc. added in excess, and boiled until all ammonia is driven off. After cooling, a few drops of phenolphthalein were added, half-normal sulfuric acid run in to slight acidity, then titrated back with alkali. The total alkali, less the amount of acid used, gives the amount of alkali required to dissolve the precipitate. Each cc. of half-normal alkali corresponds to 0.553 milligram of phosphorus.

TABLE I. PERCENTAGES OF ASH, NITROGEN AND PHOSPHORUS.

	Ash.	Fixed ash.	Inorganic ash.	N	P	N ash-free.	P ash-free.	Ratio N:P
Cell substance.....		8.61		10.65	2.87			
Immunizing portion	33.25		26.08	5.56	2.34	7.52	3.99	2.38
Prep. A.....	26.76	20.36	15.66	6.76	3.61	8.02	4.28	1.87
" B.....	35.34		30.74	4.87	1.50	7.03	2.16	3.25
" D.....	15.38	15.05	8.48	4.95	2.25	5.41	2.46	2.2
" G.....	6.99		1.66	4.65	1.74	4.73	1.77	2.67
" G purified...	5.5	5.5	1.36	3.43	1.35	3.48	1.37	2.53
" H.....	35.91	14.00	27.67	5.98	2.68	8.27	3.71	2.23
" K_2.....	7.57		2.08	5.5	1.79	5.62	1.83	3.07
" M.....	11.71	11.71	3.74	3.16	2.47	3.28	2.70	1.28
" M_2.....	8.3		3.47	5.35	1.58	5.55	1.64	3.39

Explanation of Table I.

Ash, residue after heating at low redness.
Fixed ash, residue after heating to full heat of powerful burner.
Inorganic ash, ash less calculated amount of PO_4.
N, nitrogen by Kjeldahl-Gunning method.
P, phosphorus by the Neumann method.
N and P ash free, reckoned free from "inorganic ash."
N: P, quotient of column 4 divided by column 5.
A, portion of immunizing substance dissolved by acid alcohol.
B, portion of immunizing substance not dissolved by acid alcohol.
D, substance precipitated by acid alcohol from solution of B in aqueous alkali.

[1] Treadwell: *Lehrbuch der Analytischen Chemie*, 2d ed., ii, p. 302.
[2] Koch and Woods: "Estimation of Lecithans," this *Journal*, i, p. 206, 1906.

G, substance precipitated by acid alcohol from aqueous solution of immunizing portion of colon cellular substance.

H, obtained by concentrating the alcoholic filtrate from G.

K, substance precipitated by dilute acetic acid from aqueous solution of immunizing portion of germ.

K_2, same as K, except that strong acid was used.

M, precipitated by alcohol from filtrate from K.

M_2, precipitated by alcohol from filtrate from K_2.

The large amount of ash in some of the above preparations doubtless includes the sodium salts of various decomposition products, in so far as they are insoluble in alcohol, as well as practically all the inorganic matter of the whole germ substance, both that which is an integral part of the cell, and that which is mechanically carried down from the culture medium.

As these preparations are all mixtures, the absolute values found are worth nothing taken singly, butt he comparative values, especially the ratio of N : P as given in the last column, are of interest. The determinations were made for the sake of tracing the nucleo-compounds. There are many indications of nucleic acid, but the amount of both nitrogen and phosphorus is much too small. The ratio between them is, however, quite within the range found for nucleic acids from other sources, as may be seen by comparison with the following table. Moreover the nucleic acid and the nucleates are the only nucleo-compounds

TABLE II. PERCENTAGES OF NITROGEN AND PHOSPHORUS IN NUCLEO-COMPOUNDS.

	Source.	Observer.	N	P	N : P
Nucleic acid	Sperma salmon	Miescher*	15.24	9.62	1.58
" "	" sea-urchin	Mathews	15.34	9.59	1.6
" "	Yeast	Miescher	16.03	9.04	1.77
" "	Pancreas	Bang	18.2	7.67	2.37
" "	Thymus	Kostytschew	15.55	9.25	1.69
" "	"	Bang	15.26	9.3	1.65
" "	Wheat embryo	Osborne and Harris	15.88	8.7	1.83
Inosinic acid	Muscle	Haiser	16.	8.6	1.86
Clupein nucleate		Mathews†	21.06	6.07	3.48
Nucleo-histone	Thymus	Huiskamp	18.37	3.7	4.97
Nucleo-proteid	"	"	16.42	0.95	17.3
"	Pancreas	Umber	17.82	1.67	10.65
Nuclein from above		"	17.39	4.48	3.88
Ba α-nucleate	Thymus	Kostytschew‡	12.83	7.63	1.68
Ba β-nucleate	"	"	10.16	8.48	1.38

* Mann, *Chemistry of the Proteids*, p. 442, 1906, where will be found references to the orginial articles.

† *Ibid.*, p. 454.

‡ Kostytschew: *Zeitschr. f. physiol. Chem.*, xxxix, p. 556.

in which the ratios are at all comparable with those given in Table I. Nuclein contains a little less phosphorus than any of these preparations from the germ, while other nucleo-compounds are much richer in nitrogen and poorer in phosphorus. It is perhaps worthy of mention that contact with mineral acid apparently breaks up the nucleic acid, the phosphoric acid going into solution; thus Preparation A gives evidence of phosphorus in inorganic combination, while G does not.

CARBOHYDRATES. In all these preparations qualitative tests point to the presence of carbohydrates, especially pentose. As the easiest and quickest way to obtain comparative data, samples were hydrolyzed and titrated with Fehling's solution. Gram-samples of the immunizing portion of the germ were dissolved in water containing a little alkali, neutralized, hydrochloric acid added to definite strength, and heated on the water bath in a flask with reflux condenser. The hydrolyzed solution was neutralized and titrated with Fehling's solution. Although there is undoubtedly some pentose present, there is no proof that the reducing substance is all carbohydrate. However for purposes of comparison the reducing substance was calculated as xylose. In order to find conditions giving the maximum yield, amount and strength of acid, as well as time of boiling, were varied as given in Table III.

TABLE III. REDUCING POWER OF IMMUNIZING PORTION.

No. of sample.	Amount of HCl cc.	Strength of HCl per cent.	Hours boiled.	Percentage calculated as xylose.
1	26	1	1	7.05
2	38.5	2.5	1	16.45
3	38.5	2.5	2	21.56
4	38.5	2.5	4	23.12
5	72	2.5	3	23.93
6	72	2.5	9	23.53

As shown by the table, the maximum amount of reducing substances was obtained by using 2.5 per cent acid, and boiling for three hours. Longer heating changes the results very little.

Samples of G were also hydrolyzed and titrated with results as follows:

1 gram boiled 2½ hrs. with 72 cc. of 2.5 per cent HCl gave 38.63 per cent reducing substance calculated as xylose.

1 gram boiled 5 hrs. with 72 cc. of 2.5 per cent HCl gave 43.77 per cent reducing substance calculated as xylose.

DISTRIBUTION OF NITROGEN.[1] One gram samples of the immunizing portion of the colon germ were suspended in 25 cc. of water, and 25 cc. of strong hydrochloric acid added. After standing 24 hours, they were boiled on a sand bath with a reflux condenser for 9½ hours. The biuret test was applied to one with negative results. The others were concentrated on a water bath, water and cream of magnesia added, and the ammonia distilled off into standard sulfuric acid. This is reported as amid nitrogen. The residues left in the flasks after distillation were filtered with suction and thoroughly washed. The nitrogen was determined in the precipitates, and is reported as humin nitrogen. The filtrates were treated with sulfuric and phosphotungstic acids. The nitrogen of the phosphotungstic precipitates appears in the table as diamino nitrogen, that of the filtrates as monamino. Samples of G were treated in the same way. The difficulty of determining nitrogen in the presence of the large amount of phosphotungstic acid in the monamino fraction is such that no great dependence is to be placed upon that result. Still it is useful as a check upon the whole. In the table, column 4 gives the sum of the nitrogen found in the four fractions, while column 5 gives values found directly.

TABLE IV. DISTRIBUTION OF NITROGEN.

	Amid N per cent	Humin N per cent	Monamino N per cent	Diamino N per cent	Total N cal. per cent	Total N det. per cent
Imm. portion, sample 1..	0.860	0.707	3.35	1.33	6.24	5.56
" " " 2..	0.846	0.608	3.28	1.39	6.12	
G sample 1.....	0.384	0.441	1.81	0.966	3.60	
" 2.....	0.355	0.540	1.78	0.917	3.59	3.43
Per cent of Total N.						
Of imm. portion.....	13.8	10.6	53.7	22.		
Of G.....	10.7	12.2	50.3	26.8		

ANIMAL EXPERIMENTS. The immunizing power of some of these cleavage products was tested as follows:

[1] Hausmann: *Zeitschr. f. physiol. Chem.*, xxvii, p. 95, 1899.

400 mg. of K was suspended in 30 cc. of water, and neutralized with solid sodium bicarbonate, making a fairly clear solution. Guinea pigs were inoculated intra-abdominally with 5 cc. of this solution. Thus each received 66.6 mg. of substance. Three days later each received 100 mg., and six days later four received 125 mg. in 8 cc. of water. These four died in one or two days, post mortem findings proving that death was due to local irritation, not to toxic action. The other two received on the seventh day 125 mg. of M_2. Other guinea pigs were inoculated with solutions of M and M_2, prepared in the same way. April 12, 1906, one animal from each set was inoculated with twice the fatal dose (2 cc.) of a colon culture 16 hours old. A control was dead the next morning, all the others recovered. Animals receiving twice the fatal dose on April 14 and 15 recovered. On April 16 animals succumbed to this amount, the immunity having partially worn off. On April 17 and 18, animals recovered after receiving 1 cc. of the germ culture. These results are tabulated in Table V.

TABLE V. IMMUNITY EXPERIMENTS.

No.	April 4	April 7.	April 10.	Colon culture cc.	Result.
1	66 mg K	100 mg K	125 mg K		Died April 11
2	"	"	"		" " 12
3	"	"	"		" " 12
4	"	"	"		" " 12
5	"	"	125 mg M_2	Apr. 12, 2	Recovered.
6	"	"	"	" 14, 2	"
7	66 mg M	100 mg M	116 mg M	" 12, 2	"
8	"	"	"	" 14, 2	"
9	"	"	"	" 15, 2	"
10	"	"	"	" 16, 2	Dead 7 a.m., April 17
11	"	"	"	" 17, 1	Recovered.
12	"	"	"	" 18, 1	"
13	66 mg M_2	100 mg M_2	125 mg M_2	" 12, 2	"
14	"	"	"	" 14, 2	"
15	"	"	"	" 15, 2	"
16	"	90 mg M_2	"	" 16, 2	Dead April 17.
17	"	"	"	" 17, 1	Recovered.
18	"	100 mg M_2	"	" 18, 1	"

On January 17, 1907, seven guinea pigs were inoculated with 50 mg. of Preparation G, carefully purified by repeated solution and precipitation. On January 21 one of these received 3 cc. of colon culture, was quite sick within half an hour, but soon recovered, was given 50 mg. of G on January 24, with no apparent effect. The other six pigs on January 21 received 50 mg. each of G. On January 24, one of these received 3 cc. colon culture, and 15 minutes later 50 mg. G, with same result as the preceding. A third animal received 4 cc. of colon culture, was very sick, but recovered. The other four animals received a third dose of 50 mg. of G, and at varying intervals were given 4 and 5 cc. of germ culture, but were not able

to withstand that amount. All colon cultures used were virulent ones of which the fatal dose was 1 cc.

Thus it will be seen that all these preparations confer a certain amount of immunity, but not so much as does the whole of the immunizing fraction of the cellular substance. All of them have been subjected to the action of acid, and it may be that the immune body has undergone chemical change, or it may be that the larger part is to be sought in some other portion of the haptophorous fraction.

SUMMARY AND CONCLUSIONS. By the action of sodium hydrate and alcohol, part of the proteid of the bacterial cell goes into solution in the alcohol, and part of the proteid remains undissolved. The alcoholic solution contains the poison of the cell, while the insoluble portion includes carbohydrate, nucleo-compounds, and immunizing substance.

Although the germ substance is entirely dissolved by successive treatment with dilute aqueous acid and alkali, only about 10 per cent of the portion insoluble in alkaline alcohol is dissolved by acid alcohol. And this is made up largely of inorganic salts, including considerable phosphorus separated from nucleo-compounds.

An aqueous solution of the immunizing portion of the germ was treated with acetic acid in the hope of separating mucin, but it did not give a sharp separation. The precipitate was not mucin, but resembles guanylic acid in its difficult solubility, especially in acetic acid.

Preparations precipitated by acid and alcohol resemble nucleic acids in appearance and properties. The amounts of nitrogen and phosphorus are far too small, but the relative amounts correspond very well with proportions found in nucleic acids from other sources. The failure of the biuret test shows that the proteid has been gradually broken up and it would seem that the acid used gradually decomposes the nucleic acid, phosphorus appearing in the acid alcoholic solutions as phosphate, while the alcoholic precipitates contain phosphorus in organic combination only. Decomposition is slow, for 19 grams of Preparation G yielded 16 grams after being twice dissolved and re-precipitated. There was no evidence of inorganic phosphorus in the final precipitate.

Most of the reducing substance from the non-poisonous portion of the germ is to be found in the acid precipitate G. Reckoned as xylose, since the pentose tests are very marked, we have 23.93 per cent in the material, and 43.77 per cent in Preparation G. Bang reports[1] 27 per cent of carbohydrate in guanylic acid, while Osborne and Harris[2] found evidence that triticonucleic acid contains three molecules of pentose, corresponding to 32.2 per cent xylose.

Preparation G then probably contains nucleic acid, and some of the decomposition products of the same, notably pentose. Comparing figures obtained for distribution of nitrogen in this preparation and in the immunizing portion of the germ it would appear that diamino compounds are in excess in the former.

These preparations, containing nucleic acid, possess immunizing power. Work has not progressed far enough to show that one depends upon the other, but the fact is of interest in view of the use of nuclein as a curative agent. Casein, containing only paranuclein, gives no sensitizing body.[3]

In conclusion I wish to express my thanks to Dr. V. C. Vaughan for his valuable suggestions during the progress of the work.

[1] *Loc. cit.*

[2] Osborne and Harris: "Die Nucleinsäure des Weizenembryos," *Zeitschr. f. physiol. Chem.*, xxxvi, p. 85, 1902.

[3] Vaughan and Wheeler: "Effects of Egg-White upon Animals," *Journ. of Infect. Dis.*, iv, p. 476, 1907.

A STUDY OF THE COMPARATIVE CHEMICAL COMPOSITION OF THE HAIR OF DIFFERENT RACES.

By THOMAS A. RUTHERFORD and P. B. HAWK.

(*From the Laboratory of Physiological Chemistry of the Department of Medicine of the University of Pennsylvania.*)

(Received for publication, September 27, 1907.)

I. INTRODUCTION.

Apparently the first authentic observations upon the chemical composition of hair were made by Vauquelin[1] and reported in 1806. He found that upon dissolving the hair in superheated

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steam or in mineral acids a transparent solution resulted which could be precipitated by an infusion of nut galls and which possessed the further property of blackening silver salts. He concluded that hair contained silica, carbonates, iron, manganese, calcium phosphate, and finally a considerable quantity of sulphur. Vauquelin found a lower percentage of sulphur in black hair than was present in white, blond, or red hair.

The investigations of v. Laer[1] upon the chemical composition of human hair are widely quoted. He could detect no carbonate or manganese in the hair but, like Vauquelin, he found a high sulphur content. v. Laer found the percentage of sulphur in the hair of adult males to vary from 4.63 to 5.44.

Kühne and Chittenden[2] claim that neurokeratin has a higher content of carbon and hydrogen and a lower content of nitrogen and sulphur than the keratin prepared from the hair of the white rabbit.

v. Bibra[3] found only 3.83 per cent of sulphur in the hair of a boy ten years old whereas the red hair of a man of thirty contained 8.23 per cent. The same investigator found the average sulphur content of fifteen samples of human hair to be 4.62 per cent. He further found 3.7 per cent of sulphur in the hair of a Peruvian interred four hundred years and 4.4 per cent in the hair of a Bolivian interred one thousand years. As a result of a series of analyses of the hair of different animals v. Bibra determined that 4.25 per cent was the average sulphur content.

Mohr[4] found the hair of adult females to contain 4.95 per cent of sulphur whereas that of a girl nine years of age contained 5.34 per cent. In the hair of a boy four years old Mohr found 4.98 per cent of sulphur, while the red hair of a boy six years of age contained 5.32 per cent.

II. SOURCES OF THE KERATIN ANALYZED.

Our investigation included the analysis of the hair of the following races: indian, caucasian, negro and japanese. The specimens of indian hair, ten in number, were furnished through

[1] v. Laer: *Ann. d. Chem. u. Pharm.*, xlv, p. 147, 1843.
[2] Kühne and Chittenden: *Zeitschr. f. Biol.*, xxvi, p. 291, 1890.
[3] v. Bibra: *Ann. d. Chem. u. Pharm.*, xcvi, pp. 289–302.
[4] Mohr: *Zeitschr. f. physiol. Chem.*, xx, pp. 403–406, 1895.

the courtesy of W. A. Mercer, Captain 7th Cavalry, Superintendent of the Indian Industrial School, Carlisle, Pa., and were obtained from students at the school. The negro hair, with the exception of samples 1–5, was kindly furnished us by Dr. M. M. Waldron, of the Hampton Institute, Hampton, Va. The hair of the living adult caucasians was obtained from various students of the University of Pennsylvania, and the children's hair was obtained through the courtesy of Richard Binder, Jr., of Philadelphia. The japanese hair was courteously furnished us by Mr. Okumura of the University of Pennsylvania.

The purpose of our investigation was to determine whether there were any differences in the chemical composition of the hair of the various races mentioned above. The samples of hair analyzed varied in color from an extremely light yellow to jet black and the ages of the individuals from whom the samples were obtained ranged from two and one-half years to sixty years. The average data for the hair of the different races are shown in Table X, p. 488.

III. PREPARATION OF THE HAIR FOR ANALYSIS.

The hair, after being thoroughly washed in distilled water, was placed in a large volume of artificial gastric juice and kept at 40° C. for forty-eight hours. The digestion mixture was then thrown upon cheese cloth and after draining thoroughly the hair was washed free from gastric juice by means of distilled water. The gastric fluid was now replaced by artificial pancreatic juice and the mixture again placed at 40° C. for forty-eight hours. At the end of this period the hair was removed from the pancreatic juice and washed with distilled water as before. It was now introduced into a round-bottomed flask, sufficient alcohol added to cover the sample, the flask provided with a reflux condenser and the mixture boiled on a safety water-bath for thirty-six hours. The alcohol was now removed by filtration and the hair boiled in ether for twenty-four hours. The sample was then removed from the ether, cut into exceedingly short lengths by means of scissors, and placed at 100° C. for four hours. It was then transferred to glass stoppered bottles ready for analysis.

[3] The authors wish to express their thanks to Mr. John Thomson, who kindly assisted in the preparation of the hair for analysis.

IV. METHODS OF ANALYSIS.

Sulphur was determined after fusing with sodium hydroxide and potassium nitrate. Nitrogen was determined by a modification of the Kjeldahl method in which the preliminary digestion is accomplished by means of sulphuric acid and cupric sulphate. Carbon and hydrogen were determined by Benedict's modification[1] of the Liebig method. In this modification the carbon dioxide is absorbed by soda lime and the water by sulphuric acid.

The figures for the percentage composition of the various specimens analyzed are the averages of from two to six determinations.

V. DISCUSSION OF RESULTS.

1. *Chemical composition of indian hair.* Ten samples of indian hair were analyzed, the specimens being obtained from the representatives of ten different tribes. Six of the samples were from full-blood indians and the remaining four were from half-bloods. Seven of the specimens analyzed were from female subjects and three from males. The indians whose hair was analyzed were from twelve to nineteen years of age.

The data obtained from the analysis of the ten samples of indian hair are summarized in Table I, p. 464. The lowest sulphur content (4.55 per cent) is found in the hair of the half-blood female Klamath, whereas the highest sulphur content (5.03 per cent) is found in the hair of the half-blood male Shawnee. The lowest nitrogen content (14.80 per cent) is possessed by the full-blood female Pima and the highest nitrogen content (15.72 per cent) by the half-blood female Alaskan. Carbon is present in smallest percentage (41.68 per cent) in the hair of the half-blood male Shawnee, while it is present in largest percentage (45.69 per cent) in the hair of the full-blood female Shoshone. Hydrogen follows the carbon in showing the lowest percentage (6.04 per cent) in the hair of the half-blood male Shawnee but differs from the data for carbon in showing the highest content (7.10 per cent) in the hair of the full-blood female Chippewa. The average percentage composition of the ten samples of indian

[1] Benedict: *Elementary Organic Analysis*, p. 49.

hair is given at the bottom of Table I, p. 464. By referring to these data it will be observed that the average percentage of sulphur in the ten samples of indian hair is 4.82, the average percentage of nitrogen is 15.40, the average carbon value is 44.06 per cent and the average percentage of hydrogen is 6.53.

Data for the ratio, S : N, are given in the last column of Table I, p. 464. An examination of these ratios reveals the fact that the lowest ratio, 1:3.0, is found in connection with the full-blood female Pima indian, whereas the highest ratio, 1:3.4, occurs in connection with the half-blood female Klamath indian.

2. *Chemical composition of indian hair as influenced by the sex of the individual and the purity of breeding.* A comparison from the standpoint of the sex of the individual and the purity of breeding is shown in Table II, p. 465. By referring to this table it will be noted that the two extremes of sulphur data occur in connection with the hair of the half-blood indian, *the lowest percentage (4.69) being present in the hair of the half-blood female and the highest percentage (5.03) occurring in the hair of the half-blood male indian.* On the other hand the hair of the full-blood male and female indians contains practically the same percentage of sulphur (4.85 and 4.86). Compared as to sex without reference as to whether the specimen is from half- or full-blood indians we find that the hair of the female indians contains somewhat less sulphur than that of the male indians, the percentage of sulphur in the hair of the females being 4.79 and in that of the males being 4.91. It will be further observed that, when compared as to the purity of breeding without reference to the sex, the hair of the full-blood indians contains a higher percentage of sulphur (4.86) than the hair of the half-blood indians (4.77). It is interesting to note that whereas the hair of the full-blood indians of both sexes contains practically the same percentage of sulphur (4.85 and 4.86) the hair of the half-blood female indian shows a lower percentage of sulphur (4.69) and the hair of the half-blood male indian shows a higher percentage (5.03).

By an examination of the data contained in Table II, it will be observed that the lowest percentage of nitrogen (15.20) occurs in the hair of the full-blood female indian where as the highest nitrogen content (15.60 per cent) is found in the hair of the half-blood male indian. In this connection it may be remembered

TABLE I. CHEMICAL COMPOSITION OF INDIAN HAIR.

No. of Sample.	DESCRIPTION OF SUBJECT.				PERCENTAGE COMPOSITION OF HAIR.					Ratio S : N
	Tribe.	Sex.	Age. (years)	Blood.	S	N	C	H	O	
1	Pima	Female	16	Full	4.93	14.80	43.54	6.76	29.97	1 : 3.0
2	Ute	Female	15	Full	4.83	14.88	45.56	6.56	28.17	1 : 3.1
3	Chippewa	Female	19	Full	4.79	15.64	44.83	7.10	27.64	1 : 3.3
4	Shoshone	Female	14	Full	4.88	15.48	45.69	6.59	27.36	1 : 3.2
5	Sioux	Female	16	Half	4.70	15.47	43.65	6.54	29.64	1 : 3.3
6	Alaskan	Female	18	Half	4.82	15.72	44.57	6.71	28.18	1 : 3.3
7	Klamath	Female	18	Half	4.55	15.28	45.66	6.27	28.24	1 : 3.4
8	Arapahoe	Male	14	Full	4.77	15.44	42.99	6.61	30.19	1 : 3.2
9	Pueblo	Male	17	Full	4.93	15.64	42.43	6.12	30.88	1 : 3.2
10	Shawnee	Male	17	Half	5.03	15.60	41.68	6.04	31.65	1 : 3.1
Average percentage composition of 10 samples of indian hair...					4.82	15.40	44.06	6.53	29.19	1 : 3.2

that the lowest percentage of sulphur occurs in the hair of the half-blood female indian instead of the full-blood female indian which contains the lowest percentage of nitrogen. Comparing the data obtained from the analyses of the hair of the full-blood indians we observe that the hair of the male contains considerably more nitrogen (15.54 per cent) than the hair of the female (15.20 per cent), and the same observation holds true for the hair of the half-blood indians, *i. e.*, the percentage of nitrogen in the hair of the half-blood male (15.60) is somewhat higher than the percentage of nitrogen in the hair of the half-blood female

TABLE II. CHEMICAL COMPOSITION OF INDIAN HAIR AS INFLUENCED BY THE SEX OF THE INDIVIDUAL AND THE PURITY OF BREEDING.

Sex.	Blood.	AVERAGE PERCENTAGE COMPOSITION OF HAIR.					Ratio S : N
		S	N	C	H	O	
Female	Full	4.86	15.20	44.91	6.75	28.28	1 : 3.1
Female	Half	4.69	15.49	44.63	6.50	28.69	1 : 3.3
Female	Full and half	4.79	15.32	44.79	6.65	28.45	1 : 3.2
Male	Full	4.85	15.54	42.71	6.37	30.53	1 : 3.2
Male	Half	5.03	15.60	41.68	6.04	31.65	1 : 3.1
Male	Full and half	4.91	15.56	42.37	6.26	30.90	1 : 3.2
Male and female	Full	4.86	15.31	44.17	6.62	29.04	1 : 3.2
Male and female	Half	4.77	15.52	43.89	6.39	29.43	1 : 3.3
Average percentage composition of all specimens of indian hair		4.82	15.40	44.06	6.53	29.19	1 : 3.2

(15.49). As the result of a comparison of sexes without reference to the purity of breeding it is seen that the hair of the male indians is much richer in nitrogen than the hair of the females, the percentage of nitrogen in the hair of the male being 15.56 and that in the hair of the female being 15.32. On the contrary, a comparison as to the purity of breeding without considering the sex of the individual, shows a balance of nitrogen in favor of the half-blood indians, the percentage of nitrogen in the hair of the full-blood indians being 15.31 and that in the hair of the half-bloods being 15.52.

Carbon is seen to be present in smallest percentage (41.68) in the hair of the half-blood male indian and present in the largest percentage (44.91) in the hair of the full-blood female indian, and it is very interesting to note that the order is exactly the reverse of that in which the nitrogen occurs, *i. e.*, the largest percentage of nitrogen is found in the hair of the half-blood male and the smallest percentage in the hair of the full-blood female indian. Considering the female indians apart from the males it may be observed that the carbon follows the sulphur in showing a higher percentage in the hair of the full-blood female (44.91 per cent) than in the hair of the half-blood female (44.63 per cent). In the case of the male indians however, the carbon follows a course the reverse of that taken by the sulphur and the nitrogen since it exhibits a higher percentage (42.71) in the hair of the full-blood male than is present in the hair of the half-blood male (41.68). A comparison of sexes shows the percentage of carbon in the hair of the female indians (44.79) to be considerably higher than that in the hair of the male indians (42.37) and in this point the occurrence of the carbon content again is the reverse of that of sulphur and nitrogen. Compared from the standpoint of purity of breeding, without reference to the sex of the individual, we observe that the carbon is present in larger percentage in the hair of the full-blood indians than in that of the half-bloods and in this it exhibits a similarity to the sulphur content but is the reverse of that of nitrogen. Hydrogen runs parallel with the carbon in every instance.

The relation of the sulphur content to the nitrogen content is stated in the last column of Table II, p. 465. The most striking thing observed in this connection is the fact that the ratio, S: N, for the specimens of hair from the male indians, without respect to the purity of breeding, is exactly the same as the ratio in the case of the specimens of hair from the female indians, *i. e.*, 1:3.2. Considering the female indians alone we observe that the ratio varies with the purity of breeding, being 1:3.1 for the full-blood females and 1:3.3 for the half-blood females. In the case of the male indians the ratios run differently since they show a ratio of 1:3.2 for the full-blood indians and 1:3.1 for the half-bloods. It is interesting to note that the ratio 1:3.2 is common to four of the eight classes included in the table.

3. *Chemical composition of negro hair.* Fourteen specimens of negro hair were analyzed, five of them being obtained from dead individuals and the remaining nine from living individuals. Five of the latter were full-blood negroes ranging from seventeen to twenty-three years of age, two were half-blood negroes eighteen years of age, and two were three-quarter blood negroes eighteen and twenty years old, respectively. Of the fourteen specimens of negro hair analyzed eleven were obtained from females and three from males.

In Table III, p. 469 are summarized the data from the chemical analyses of the fourteen samples of negro hair. The first impression gathered from an examination of the table is *the general similarity of the analytical results with those from the analyses of indian hair*, as set forth in Table I, p. 464. *There is also observed to be a well marked tendency for both the sulphur and the nitrogen to occur in higher percentage in the hair of full-blood negroes than in the hair of the negroes less purely bred, this higher percentage occurring irrespective of the sex of the individual. It will also be noted that the analyses of the hair of dead negroes show a rather lower average percentage of sulphur and a somewhat higher average percentage of nitrogen than is shown in the data from the analyses of the hair of the living negroes.* These three points, mentioned in this general way, are more fully discussed on pp. 470–476.

Considering the sulphur content of the various specimens of negro hair we observe that the lowest percentage (4.53) occurs in the hair of a dead female negro (sample 2), whereas the highest percentage (5.15) occurs in the hair of a living full-blood female negro (sample 8). Nitrogen follows the sulphur in being found in the largest percentage (15.55) in the sample containing the greatest abundance of sulphur but differs from it in showing the lowest percentage (14.51) in the hair of the living, three-quarter blood female negro. The lowest percentage of carbon (42.43) is found in the hair of a living male half-blood negro (sample 14), and the highest percentage (45.70) in the hair of a living, full-blood female negro (sample 6). In this connection it is exceedingly interesting to note that an examination of the data for carbon reveals the singular fact that *the hair of the impurely bred negroes, i.e., the half- and three-quarter bloods, contains, uniformly considerably less carbon than the hair of any of the other negroes,*

irrespective of the sex of the individual (see data for samples 9, 10, 11, and 14).

Hydrogen follows the carbon in showing the lowest percentage (5.60) in the hair of a living, half-blood male negro (sample 14) and the next lowest (5.97) in the hair of a living, half-blood female negro (sample 11), but differs from the carbon in the fact that it is found in greatest amount (6.90 per cent) in the hair of a living full-blood female negro (sample 7).

The average percentage composition of the fourteen samples of negro hair is given at the bottom of Table III, p. 469. From this it will be observed that the average percentage of sulphur is 4.84 and the average percentage of nitrogen is 14.90 whereas the carbon and hydrogen averages are 43.85 per cent and 6.37 per cent respectively.

The data for the ratio, S:N, in the various samples of negro hair, will be found in the last column of Table III, p. 469. Among the interesting observations in relation to this ratio is the general uniformity of the ratios for the samples of hair of the living, full-blood negroes and the decided variation of this ratio from that obtained from the data for the samples of the hair of dead negroes. The ratio for the hair of the living, full-blood individuals is 1:2.9 for two samples and 1:3.0 for three samples whereas the ratio for the hair of the dead individuals is 1:3.1 for two samples, 1:3.2 for two samples, and 1:3.4 for one sample, making a variation from 1:3.1 to 1:3.4 as contrasted with the lesser variation from 1:2.9 to 1:3.0 noted in the case of the living, full-blood negroes. The two instances in which the ratio for the living individuals is above 1:3.0 are both impurely bred negroes, one being a three-quarter-blood female and the other a half-blood male. The hair of each of these individuals shows a ratio of 1:3.2. An examination of the ratios for the various samples shows conclusively that the average ratio for the impurely bred individuals is considerably above the average ratio for the full-blood individuals. These average ratios are further discussed on p. 474. The average ratio for the entire fourteen samples of negro hair is 1:3.1.

4. *Chemical composition of negro hair as influenced by the sex of the individual and the purity of breeding.* In Table IV, p. 471, is portrayed the chemical composition of the hair of living and dead negroes as influenced by the sex of the individual and the

TABLE III. CHEMICAL COMPOSITION OF NEGRO HAIR.

No. of Samples.	DESCRIPTION OF SUBJECT.				PERCENTAGE COMPOSITION OF HAIR.						Remarks.
	Sex.	Blood.	Age. (years)	Living or Dead.	S	N	C	H	O	Ratio S : N	
1	Female		40–50	Dead	4.72	15.18	43.80	6.40	29.90	1 : 3.2	
2	Female		40–50	Dead	4.53	15.31	44.76	6.53	29.07	1 : 3.4	
3	Female		40–50	Dead	4.81	14.76	44.80	6.77	29.86	1 : 3.1	
4	Female		40–50	Dead	4.84	15.18	44.13	6.32	29.53	1 : 3.1	
5	Female		40–50	Dead	4.77	15.14	44.61	6.26	29.22	1 : 3.2	
6	Female	Full	17	Living	4.96	14.59	45.70	6.15	28.60	1 : 2.9	Composite sample, 5 specimens.
7	Female	Full	18	Living	4.95	14.76	43.75	6.90	30.44	1 : 3.0	Composite sample, 4 specimens.
8	Female	Full	19	Living	5.15	15.55	44.70	6.79	27.81	1 : 3.0	Composite sample, 3 specimens.
9	Female	Three-quarter	18	Living	4.71	14.55	43.11	6.66	30.97	1 : 3.0	
10	Female	Three-quarter	20	Living	4.56	14.51	42.90	6.45	31.68	1 : 3.2	
11	Female	Half	18	Living	5.00	14.68	42.44	5.97	31.91	1 : 2.9	
12	Male	Full	20	Living	5.13	14.93	43.43	6.20	30.31	1 : 2.9	
13	Male	Full	20 and 23	Living	4.93	14.64	43.31	6.15	30.97	1 : 3.0	Composite sample, 2 specimens.
14	Male	Half	18	Living	4.69	14.80	42.43	5.60	32.48	1 : 3.2	
Average percentage composition of 14 samples of negro hair.....					4.84	14.90	43.85	6.37	30.04	1 : 3.1	

purity of breeding. Considering first the sulphur content, one of the most striking points we observe is the similarity between the data for the living, full-blood female negroes and the living full-blood male negroes, the percentage of sulphur being 5.02 for the former and 5.03 for the latter. *The value 5.03 for the full-blood male negroes is the highest percentage of sulphur found in any of the samples of negro hair analyzed.* The lowest percentage of sulphur (4.64) is found in the hair of the living, three-quarter blood female negro and the next lowest (4.69) occurs in the hair of the living, half-blood male negro. Grouping the female and the male negroes together for purposes of comparison we observe that the average percentage of sulphur is 5.02 for the full-blood negroes and the lower value of 4.84 for the half-blood negroes. The most striking variation, however, in the sulphur content of the hair of the full-blood and half-blood negroes is noted in the case of the male subjects in which connection it will be observed that the *hair of the half-blood individuals contains only 4.69 per cent of sulphur whereas the hair of the full-blood individuals contains 5.03 per cent*, which is, as has just been mentioned, the maximum percentage of sulphur for the negro hair examined.

Comparing the data from the analyses of the hair of the dead female negroes with the data from the analyses of the hair of the living, female negroes we observe that the percentage of sulphur in the hair of the former (4.73) is somewhat lower than that in the latter (4.89). One of the most interesting points emphasized by this table is the very close agreement in percentage of sulphur shown in the analyses of the hair of living, male and female negroes without reference to the purity of breeding. In this connection it will be noted that the hair of the female negroes contains 4.89 per cent of sulphur and the hair of the male negroes contains 4.92 per cent of sulphur, the variation being but 0.03 per cent which is within the limit of experimental error for such determinations. The average percentage of sulphur in all the specimens of negro hair without reference to the sex of the individual or the purity of breeding is 4.84 and is identical with that determined for the living, half-blood male and female negroes and within the limit of experimental error of that determined for the living and dead females (4.82), for the living, full-, half- and three-quarter blood female negroes (4.89) and the living, full- and half-blood male negroes (4.92).

TABLE IV. CHEMICAL COMPOSITION OF THE HAIR OF LIVING AND DEAD NEGROES AS INFLUENCED BY THE SEX OF THE INDIVIDUAL AND THE PURITY OF BREEDING.

Sex.	Blood.	Living or dead.	AVERAGE PERCENTAGE COMPOSITION OF HAIR.					Ratio S : N
			S	N	C	H	O	
Female	Full	Living	5.02	14.96	44.72	6.61	28.69	1 : 3.0
Female	Three-quarter	Living	4.64	14.53	43.00	6.55	31.28	1 : 3.1
Female	Half	Living	5.00	14.68	42.44	5.97	31.91	1 : 2.9
Female	Full, three-quarter and half.	Living	4.89	14.77	43.77	6.49	30.08	1 : 3.0
Female		Dead	4.73	15.11	44.42	6.46	29.28	1 : 3.2
Female		Living and dead	4.82	14.93	44.06	6.47	29.72	1 : 3.1
Male	Full	Living	5.03	14.79	43.37	6.18	30.63	1 : 2.9
Male	Half	Living	4.69	14.80	42.43	5.60	32.48	1 : 3.2
Male	Full and half	Living	4.92	14.79	43.06	5.98	31.25	1 : 3.0
Male and female	Full	Living	5.02	14.89	44.18	6.44	29.47	1 : 3.0
Male and female	Half	Living	4.84	14.74	42.44	5.79	32.19	1 : 3.0
Average percentage composition of all specimens of negro hair			4.84	14.90	43.85	6.37	30.04	1 : 3.1

The most noteworthy observation in connection with the nitrogen content of the samples of negro hair is the variation of the percentage of nitrogen in the hair of the dead female negroes from the percentage of nitrogen in the hair of all the other classes of living subjects whether male, female, full-, half- or three-quarter blood. *The hair of the dead females contains 15.11 per cent of nitrogen whereas that of the other classes of subjects varies from 14.53 per cent to 14.96 per cent.* One of the most interesting points concerning the nitrogen content of the samples of negro hair is the uniformity in the percentage of this element which is present in the hair of the various classes of living subjects as grouped in Table IV, p. 471. By referring to the table it will be seen that, with the exception of the hair of the three-quarter blood female negroes, all the percentage data for the nitrogen of the hair of all the classes in the table may be grouped together as duplicates which are almost within the limit of experimental error. Of such variations as occur in these data the most striking is the tendency of the nitrogen content to follow that of sulphur in being higher in the hair of the full-blood negroes than in that of the half-bloods, *e. g.*, 14.89 per cent of nitrogen in the hair of full-bood male and female negroes and 14.74 per cent in the hair of the half-blood negroes. The average content of nitrogen for the entire fourteen specimens of negro hair analyzed is 14.90 per cent, a value which is practically duplicated by all classes in Table IV except the dead female negroes and the living, half- and three-quarter blood female negroes.

Carbon shows a well developed tendency to be present in the greatest amount in the hair of the purest bred negroes. This is most strikingly indicated in the data for the living female subjects where the hair of the full-blood individuals is shown to possess 44.72 per cent of the element, that of the three-quarter blood female 43.00 per cent and finally the half-blood's hair showing 42.44 per cent of carbon. *The series shows a decreasing carbon content as the purity of breeding is lowered, the maximum percentage occurring in the hair of the purely bred individual and the minimum in the hair of the half-blood.* This series also practically includes the two extremes of the eleven classes as grouped in Table IV, since 44.72 per cent is the highest of those tabulated and 42.44 per cent is practically the lowest since it is almost

identically the same as the value (42.43 per cent) for the carbon content of the hair of the living half-blood male negroes which is the *actual* minimum percentage of carbon. This tendency to exhibit a variation in carbon content according to the purity of breeding is shown likewise in the case of the male negroes. In this instance the hair of the full-blood individual contains 43.37 per cent of carbon while that of the half-blood contains but 42.43 per cent. Grouping the males and females together we obtain as the averages 44.18 per cent of carbon in the hair of the full-blood negroes and 42.44 per cent in the hair of the half-bloods. In this last particular the carbon follows the tendency already noted in connection with sulphur and nitrogen. Dividing the female negroes into living and dead subjects we observe that *the hair of the dead subjects contains considerably more carbon than that of the living subjects*, *e. g.*, 44.42 per cent being present in the hair of the former and 43.77 per cent in that of the latter. In this regard the carbon again follows the course of the nitrogen. Comparing the living females with the living males we note that the hair of the former contains a higher percentage of carbon than the hair of the latter, the averages being 43.77 per cent and 43.06 per cent respectively. The average percentage of carbon in the whole series of samples of negro hair was 43.85, a value most nearly duplicated by the hair of the living, female negroes (43.77), the living, full-blood male and female negroes (44.18) and the living and dead female negroes (44.06).

There is a very well defined tendency for the hydrogen to follow very closely the same scheme of distribution as that noted for the carbon content. First of all we observe the same general decrease in the percentage of hydrogen coincident with a lessening of the purity of breeding. This feature is particularly well illustrated in the case of the living, female negroes, where, it will be noted, the hair of the full-blood individuals contains 6.61 per cent of hydrogen, that of the three-quarter blood 6.55 per cent and that of the half-blood 5.97 per cent. This variation according to the purity of breeding is also well illustrated by the data for the analyses of the hair of the male negroes. Here the hair of the full-blood individual possesses a hydrogen content of 6.18 per cent as contrasted with a content of 5.60 per cent for the hair of the half-blood negro. The hydrogen again follows the carbon in showing its maximum percentage in the hair of the living, full-blood

female and its minimum percentage in the hair of the living, half-blood male. However, like the sulphur it is present in slightly higher percentage in the hair of the living, female negroes than in that of the dead female negroes. In a comparison of the living females with the living males it is noted that the hydrogen follows the carbon in being present in largest percentage in the hair of the former, the percentage being 6.49 for the hair of the females and 5.98 for the hair of the males.

The noteworthy uniformity with which the hair of the full-blood negroes, irrespective of sex, contains a higher percentage of each of the constituents determined by analysis, than the hair of the half-blood negroes is very strikingly portrayed in the lower portion of Table IV. *Sulphur, nitrogen, carbon and hydrogen are found with absolute regularity in higher percentage in the hair of the full-blood negroes than in the hair of the half-blood negroes.*

The ratio, S : N, is the lowest (1:2.9) for the living, half-blood female negroes and living, full-blood male negroes, and highest (1:3.2) for the dead female and the living, half-blood males. The average ratio for all specimens of negro hair is 1:3.1.

5. *Comparison of the chemical composition of indian and negro hair.* By careful examination of Tables II and IV, pp. 465 and 471, some interesting observations may be made upon the comparative chemical composition of the hair of the indian and negro. Referring first to the sulphur content it will be observed that the percentage limits are virtually identical in the two instances, the highest percentage of sulphur in the indian hair being 5.03 and the lowest being 4.69, whereas the similar data for negro hair are 5.03 and 4.64. It is of interest in this connection to note that in each instance *the hair of the male subject contained the higher percentage of sulphur*, one of the most striking comparative observations portrays the influence of purity of breeding upon the chemical composition of the hair of the individual. An examination of the tables will show the great uniformity with which *the hair of the full-blood male or female indian or negro is shown to contain a higher percentage of sulphur, carbon and hydrogen than the hair of the half-blood*, and, in the case of negro hair, nitrogen also. The only exception to this rule is the nitrogen content of indian hair where the hair of the impurely bred male

or female subject is, in every instance, observed to contain a higher percentage of nitrogen than that of the full-blood subject. The variation in the chemical composition according to sex is clearly shown in the case of the sulphur content also. In both tables it will be seen that the hair of the male subjects contains a higher percentage of sulphur than the hair of the female subjects, the data for indian hair being 4.91 and 4.79 and the data for negro hair being 4.92 and 4.89. The average percentage of sulphur is practically the same for each variety of hair, *e. g.*, 4.82 for indian hair and 4.84 for negro hair.

The most striking lack of uniformity is shown in the comparison of the nitrogen content. *The hair of the indian contains uniformly a higher percentage of nitrogen than that of the negro.* This fact is emphasized by an examination of the tables which reveals the fact that the lowest percentage of nitrogen determined for indian hair (15.20) is nevertheless somewhat higher than the maximum percentage of nitrogen (15.11) in negro hair. The percentage of nitrogen in indian hair varies from 15.20 to 15.60 whereas the percentage of nitrogen in negro hair varies from 14.53 to 15.11. Indian hair contains an average of 15.40 per cent of nitrogen while the average nitrogen value of negro hair is 14.90 per cent.

An examination of the data for the carbon determinations reveals another interesting similarity. It is there shown that the maximum percentage of carbon in the hair of both the indian and the negro is found in the hair of the full-blood female and the minimum percentage of carbon is found in the hair of the half-blood male. *The tendency is well developed for the hair of the full-blood individual of either race to contain a higher percentage of carbon than the hair of any individual of that race less purely bred.* The average percentage of carbon for the indian hair (44.06) is somewhat higher than the average for negro hair (43.85) but the difference is almost within the limit of experimental error for carbon determinations. Contrasted according to sex it will be observed that in each table the hair of the females contains a higher percentage of carbon than that of the males.

Hydrogen follows carbon in the main characteristics. For instance *we note the same marked tendency for the percentage of hydrogen to decrease as the purity of breeding is lowered, as well as*

the strict uniformity with which the sex appears to a degree to regulate the chemical composition of the hair. Thus we find that the hair of the full-blood individuals contains a higher percentage of hydrogen than that of the half-blood individuals, and it is also noted in every instance, that the hair of the females contains a higher percentage of hydrogen than that of the males. In common with carbon, hydrogen is also present in maximum percentage (6.75) in the hair of the full-blood female indian and in minimum percentage (5.60) in the hair of the half-blood male negro. The average percentage of hydrogen for the whole series of samples of indian hair (6.53) is somewhat higher than the similar average for negro hair (6.37).

The ratio, S : N, for the indian hair is higher throughout than the same ratio for the negro hair. This is due to the uniformly higher nitrogen content of the former.

TABLE V. COMPARATIVE CHEMICAL COMPOSITION OF THE HAIR OF FULL-BLOOD MALE INDIANS, NEGROES AND JAPANESE.

Subject.	PERCENTAGE COMPOSITION OF HAIR.					Ratio S : N
	S	N	C	H	O	
Negro	5.03	14.79	43.37	6.18	30.63	1 : 2.9
Japanese	4.96	14.64	42.99	5.91	31.50	1 : 3.0
Indian	4.85	15.54	42.71	6.37	30.53	1 : 3.2

6. *Chemical composition of japanese hair.* Unfortunately but a single sample of japanese hair was available for analysis, this being the hair of a full-blood male japanese. The data for the analysis of this specimen are given in Table V, p. 476, in which table are also given, for the purpose of comparison, the average data for the chemical composition of the hair of full-blood male indians and negroes. It is rather interesting to note the similarity in the chemical composition of these samples of hair from three distinct races, in fact, *the agreement of the data, with the exception of the nitrogen content, is as close as that previously noted as existing in some instances between the data for different sexes of the same race, or between those noted for full-blood and half-blood individuals of the same sex.* Considering, for example, the sulphur content, we observe that the percentage of this element in the japanese hair is an approximate mean of the percentage of

sulphur in the hair of the indian and the percentage of sulphur in the hair of the negro, being a slightly lower percentage than that obtained from the analysis of negro hair and slightly higher than the determined percentage of sulphur in indian hair. We do not note the same uniformity in the nitrogen content of the three varieties of hair. There is rather close agreement between the data for negro and japanese hair, the percentage in the former case being 14.79 and in the latter case 14.64, but the percentage of nitrogen in the hair of the indian is considerably higher (15.54), in fact, as may be seen from a comparison of Tables II and IV, *the percentage of nitrogen is uniformly high for all the specimens of indian hair as compared with the similar data for the negro hair.* In connection with the carbon content the same conditions obtain as those noted in the discussion of the sulphur content, *i. e.*, the percentage of carbon in the hair of the japanese is an approximate mean of the similar data for the negro hair and indian hair, being somewhat lower than the percentage of carbon in the hair of the negro and somewhat higher than the percentage of carbon in the indian hair. The percentage of hydrogen is similar to that of nitrogen in showing the highest percentage (6.37) in the hair of the indian and the lowest (5.91) in the hair of the japanese, the percentage of hydrogen in the hair of the negro being an approximate mean of those percentages. The ratio, S : N, is lowest (1:2.9) for the hair of the negro, slightly higher (1:3.0) for the hair of the japanese, while the maximum ratio (1:3.2) occurs in connection with the indian hair.

7. *Chemical composition of caucasian hair.* There were, in all, twenty specimens of caucasian hair subjected to analysis, thirteen of which were obtained from adults. The remaining seven specimens were the hair of children ranging from two and one-half to twelve years of age. In order to be able to interpret the results more satisfactorily we will consider the analyses of the caucasian hair in two divisions, *i. e.*, adults and children.

(*a*) *Chemical composition of the hair of adult caucasians.* Thirteen specimens of hair obtained from adult caucasians were analyzed, five of them being from dead females and eight from living males.

The data for the analyses of the hair of adult caucasians are given in Table VI, p. 478. Referring to this table and consider-

TABLE VI. CHEMICAL COMPOSITION OF THE HAIR OF ADULT CAUCASIANS.

No. of Sample.	Color of Sample.	DESCRIPTION OF SUBJECT.			PERCENTAGE COMPOSITION OF HAIR.					Ratio S : N
		Sex.	Age.	Living or Dead	S	N	C	H	O	
1	Gray	Female	Old	Dead	4.94	15.84	44.75	6.24	28.23	1 : 3.2
2	Gray	Female	Old	Dead	5.00	15.22	44.66	6.30	28.82	1 : 3.0
3	Gray	Female	Old	Dead	4.74	14.97	43.96	6.36	29.97	1 : 3.2
4	Brown	Female	Young	Dead	4.82	14.85	44.02	6.55	29.76	1 : 3.1
5	Brown	Female	Young	Dead	4.49	14.70	44.83	6.67	29.31	1 : 3.3
6	Light	Male	22	Living	5.27	15.31	44.44	6.62	28.36	1 : 2.9
7	Light	Male	24	Living	5.69	15.31	44.41	6.25	28.34	1 : 2.7
8	Light	Male	21	Living	5.63	15.35	43.15	6.25	29.62	1 : 2.7
9	Light	Male	23	Living	4.72	14.93	43.47	6.11	30.77	1 : 3.2
10	Brown	Male	27	Living	5.26	15.56	45.79	6.37	27.02	1 : 3.0
11	Dark brown	Male	25	Living	5.51	15.18	44.67	6.66	27.98	1 : 2.7
12	Red	Male	26	Living	6.07	15.19	45.18	6.66	26.90	1 : 2.5
13	Red	Male	26	Living	5.73	15.11	45.07	6.66	27.43	1 : 2.6
Average percentage composition of 13 samples of caucasian hair.					5.22	15.19	44.49	6.44	28.66	1 : 2.9

ing first the sulphur determinations we are struck at once by the general tendency of the percentage of sulphur in these samples to be much higher than the percentage of sulphur in the hair of any of the races already considered. The maximum percentage for the series is 6.07 which was found in the hair (red) of a living male twenty-six years of age, whereas the minimum percentage for the series is 4.49 which was found in the hair (brown) of a dead female of unknown age. This tendency for the higher percentages of sulphur to be found in the hair of the living males is quite well marked as will be seen from an examination of the table. This point is more fully discussed on p. 482. *The fact that each of the samples of red hair analyzed contained a higher percentage of sulphur than any of the other samples of hair, all races and sexes included is particularly worthy of note.* The nitrogen determinations, as a rule, are seen to be higher than they were in the case of the negro hair and lower than were found in the indian hair. The maximum percentage of nitrogen (15.84) is found in the hair (gray) of a dead female of unknown age, whereas the minimum percentage of nitrogen (14.70) occurs in the hair (brown) of a dead female of unknown age. After examining the data for the carbon we note that they follow the sulphur in showing the same well marked tendency to be higher than the similar data for the hair of any of the other races examined. The maximum percentage of carbon is 45.79 which occurs in the hair (brown) of a male twenty-seven years of age, whereas the minimum percentage (43.15) occurs in the hair (light) of a male twenty-one years of age. There is no striking difference in the general trend of the percentage of hydrogen in the specimens of caucasian hair from the percentages as noted for the hair of the three other races examined.

The average percentage composition of the thirteen samples of the hair of adult caucasians is given at the bottom of Table VI, p. 478. It will be noted that the average percentage of sulphur for this variety of hair is higher than that of any of the other varieties of hair examined. This value for caucasian hair is 5.22 per cent and is followed in descending sequence by japanese hair (4.96 per cent), negro hair (4.84 per cent) and indian hair (4.82 per cent). The average percentage of nitrogen is 15.19, a percentage which is exceeded only by the percentage of nitrogen

in the hair of the indian (15.40). The similar data for negro hair is 14.90 per cent and for japanese hair 14.64 per cent. In common with the data for the percentage of sulphur the average percentage of carbon in the hair of adult caucasians is higher than that for the hair of the three other races considered, the caucasian hair containing an average of 44.49 per cent of carbon and being followed in descending sequence by indian hair (44.06), negro hair (43.85), and japanese hair (42.99). The average percentage of hydrogen in the caucasian hair is 6.44 and is higher than the percentage obtained for japanese hair (5.91) and negro hair (6.37) but is lower than the average of hydrogen in indian hair (6.53).

The ratio, S : N, undergoes more pronounced variations in the analyses of caucasian hair than were observed in the data for the analyses of the hair of other races. This ratio varies from 1:3.3 to 1:2.5, the higher ratio being that obtained from the analytical data for the hair (brown) of a female of unknown age and the lower ratio being possessed by the hair (red) of a male, twenty-six years of age. The next lower ratio, 1:2.6, also occurs in connection with the red hair of a male twenty-six years of age. The ratios 1:2.5, 1:2.6 and 1:2.7 are lower ratios than were obtained for any of the specimens of hair of the other races. The average ratio for the thirteen samples of hair of caucasian adults is 1:2.9 and is the same ratio as was determined for the hair of the full-blood male negroes and is lower than the average ratios for the hair of the japanese (1:3.0) and the indian (1:3.2).

From what has gone before it will be observed that the data for the average percentage composition of the thirteen samples of caucasian hair show a well marked tendency for the average values to run higher for sulphur, carbon and hydrogen than the similar data for the hair of the other races mentioned. The percentage of nitrogen is also high, being exceeded alone by the percentage of nitrogen in indian hair.

(*b*) *Chemical composition of the hair of adult caucasians as influenced by the color of the hair and the sex of the individual.* Data showing the influence of the color of the hair and the sex of the individual upon the chemical composition of the hair may be found in Table VII, p. 481. The most striking point brought out in this table is the pronounced variation in the sulphur con-

TABLE VII. CHEMICAL COMPOSITION OF THE HAIR OF ADULT CAUCASIANS AS INFLUENCED BY COLOR OF THE HAIR AND THE SEX OF THE INDIVIDUAL.

Sex.	Color of Hair.	Living or Dead Subject.	Average percentage composition of hair.					Ratio S : N
			S	N	C	H	O	
Female	Gray	Dead	4.89	15.34	44.46	6.30	29.01	1 : 3.1
Female	Brown	Dead	4.66	14.77	44.42	6.61	29.54	1 : 3.1
Female	All specimens	Dead	4.80	15.12	44.44	6.42	29.22	1 : 3.1
Male	Light	Living	5.33	15.23	43.87	6.31	29.26	1 : 2.8
Male	Brown	Living	5.26	15.56	45.79	6.37	27.02	1 : 3.0
Male	Dark brown	Living	5.51	15.18	44.67	6.66	27.98	1 : 2.7
Male	Red	Living	5.90	15.15	45.13	6.66	27.16	1 : 2.6
Male	All specimens	Living	5.49	15.24	44.52	6.44	28.31	1 : 2.8
Average percentage composition of all specimens.....			5.22	15.19	44.49	6.44	28.66	1 : 3.0

tent of the samples analyzed. As will be seen by an examination of Tables I and III, pp. 464 and 469, the percentage variation in the amount of sulphur in the hair of the indian and negro is only about 0.5 per cent (0.48 per cent for indian hair and 0.62 per cent for negro hair), whereas here in the case of the hair of the caucasian adults the percentage of sulphur varies from 4.66 per cent to 5.90 per cent, a variation of 1.24 per cent. The brown hair of dead females contains the smallest percentage (4.66) of sulphur, whereas the red hair of living males contains the largest percentage (5.90). Another very interesting point shown by the analyses is the *high sulphur content of the hair of the males as compared with the hair of the females the average percentage of sulphur in the hair of the males being 5.49 and the similar datum for the hair of the females being 4.80.* It must be remembered, however, that the hair of the females was all obtained from dead subjects whereas that of the males was taken from the living subjects. Unfortunately we have no data obtained from the analyses of the hair of living females or dead males for comparison. Work along these lines is contemplated. However, we are inclined to believe that the color of the hair, the age and sex of the individual as well as the condition of the individual when the sample of hair is obtained, *i. e.*, living or dead, contribute to influence the chemical composition. From an examination of Table III, p. 469, it will be seen that the data there recorded seem to indicate that the samples of negro hair obtained from the dead subjects contain rather less sulphur than those samples which were obtained from the living individuals of the same sex.

Considering the sexes separately we find that *the highest percentage (4.89) of sulphur in the hair of the females is present in the samples of gray hair, and the lowest percentage (4.66) in the brown hair.* In the case of the samples of hair from the males the minimum percentage (5.26) of sulphur is found in the brown hair and the maximum percentage (5.90), as already mentioned, occurs in the red hair.

An examination of the data for the percentages of nitrogen in the hair samples fails to reveal the pronounced and uniform variation between the specimens of hair of the two sexes which was observed in the case of the sulphur. However, the brown hair of the males contains the maximum percentage of nitrogen

(15.56) whereas the brown hair of the females contains the minimum percentage of nitrogen (14.77). Taking the average nitrogen values for each sex separately, however, we find that the males show an average nitrogen value of 15.24 per cent and the females show an average of 15.12 per cent. There are no noteworthy variations to be observed among the nitrogen data for the samples of caucasian hair.

The data for carbon and hydrogen follow the nitrogen in being devoid of any pronounced variations. The average percentage of carbon in the hair of the females is 44.44 and the similar value for the hair of the males is 44.52. In the case of hydrogen the agreement is much closer being 6.42 per cent and 6.44 per cent for the hair of the females and males, respectively.

The ratio, S : N, appears to run on a lower plane for the caucasian hair than for the specimens of hair obtained from the representatives of the other races mentioned. The ratio for the hair of the females is uniformly 1:3.1, whereas the ratio for the hair of the males varies from 1:2.6 to 1:3.0 the minimum ratio occurring in connection with the red haired individuals and the maximum ratio occurring in connection with the brown haired individuals. Comparing the individuals by sex it will be seen that the average ratio for the females is 1:3.1 and the average ratio for the males is the rather lower ratio 1:28. It has already been pointed out that the percentage of nitrogen in the hair of the two sexes does not vary to any considerable degree, therefore this rather wide variation in the ratio, S : N, shown by the data for the analyses of the specimens of hair of the two sexes must be due, almost entirely, to the pronounced and uniform tendency of the hair of the males to contain a quantity of sulphur notably in excess of that contained in the hair of the females.

(*c*) *Chemical composition of the hair of caucasian children.* Seven samples of this variety of hair were analyzed, six of them being from females. Three of the samples from females were composite samples made up of from four to twelve different specimens of hair, the single sample of male hair was also a composite sample, being composed of hair from nine different individuals. Those from whom the various specimens of children's hair were obtained ranged from two and one-half to twelve years of age. Analytical data for these samples of hair

may be seen in Table VIII, p. 485. Taking the table as a whole the most notable feature is the somewhat lower values obtained for the various constituents of the hair as contrasted with the similar data (Table VI) for the hair of the caucasian adults. This point is especially to be noted in the data for sulphur, nitrogen and carbon.

An examination of the data for sulphur fails to show any such variation according to the sex as is shown in the examination of the hair of adults (Table VI). In the case of the children we observe that the sulphur values range from 4.77 per cent to 5.10 per cent, a difference of only 0.33 per cent as contrasted with a variation of 1.24 per cent which is noted in the discussion of the data from the analyses of the hair of adults. *Here for the first time we find the sulphur content of the hair of the male to be lower than that of the female.* In all the other tables, as will be seen by an examination, the data show the females to possess hair of a lower sulphur content than that of the males. With the caucasian children, however, this order is reversed and *the lower sulphur content is shown to be possessed by the hair of the males.* It should be mentioned, however, that this conclusion is based upon the analysis of a *single sample of boys' hair* and that further analyses of other specimens of hair may serve to show the average sulphur content of such samples to be higher than that of the samples of hair from the females and thus conform with the data of the analyses of the samples of hair of individuals of other races. However, the dependability of the data from the analysis of this single sample is enhanced from the fact that it was a composite sample containing hair from nine different individuals ranging from three to seven years of age. Another point to be borne in mind, in this connection, is the fact that the samples of hair under consideration were obtained from individuals much younger than those from whom the other samples of hair were obtained. It may therefore be possible that the relative chemical composition of the hair changes with the development of the individual and that in childhood the hair of the male may contain less sulphur than that of the female, but that the condition is reversed with the maturing of the individual and that later in life the hair of all races would show uniformity in that the hair of the male would contain the larger percentage of sulphur. It will be

TABLE VIII. CHEMICAL COMPOSITION OF THE HAIR OF CAUCASIAN CHILDREN.

No. of Sample.	Color of Sample.	Description of Subjects.		Percentage Composition of Hair.					Ratio S : N	Remarks.
		Sex.	Age. (years)	S	N	C	H	O		
1	Auburn	Girl	7	4.80	14.56	43.87	6.26	30.51	1 : 3.0	
2	Dark brown	Girl	7	5.05	14.68	44.34	6.66	29.27	1 : 2.8	
3	Dark brown	Girl	3	5.10	14.64	41.65	6.18	32.43	1 : 2.9	
4	Dark brown	Girls	6 to 9	4.83	14.38	43.21	6.90	30.68	1 : 3.0	Composite sample, 4 specimens.
5	Dark brown to red	Girls	4 to 12	5.03	14.52	43.26	6.57	30.62	1 : 2.9	Composite sample, 12 specimens.
6	Light brown to dark brown	Boys	3 to 7	4.77	14.76	43.56	6.22	30.69	1 : 3.1	Composite sample, 9 specimens.
7	Light	Girls	2½ to 9	4.93	14.51	42.72	6.40	31.44	1 : 3.0	Composite sample, 9 specimens.
Average percentage composition of 7 samples of children's hair.				4.93	14.58	43.23	6.46	30.80	1:3.0	

remembered that Mohr[1] found a lower percentage of sulphur in the hair of a boy of four than in the hair of a girl of nine.

Two striking points are brought out in connection with the data for nitrogen, as shown in Table VIII, p. 485. It will be noticed that the nitrogen percentage in these samples of hair is uniformly lower than that of any of the other forms of hair examined, those forms of hair most nearly approaching it in point of nitrogen content being the hair of the japanese and the negro. The second point of interest is the close agreement in the data for the nitrogen content of the various samples. The minimum percentage of nitrogen (14.38 per cent) is found in the composite sample of the dark brown hair of girls who were from six to nine years of age, and further, this is not only the minimum percentage of nitrogen for the hair of caucasian children but for each of the varieties of hair investigated. The maximum percentage of nitrogen (14.76) is found in the composite sample of the brown hair of boys who were from three to seven years of age. The close agreement of the nitrogen values obtained from the various samples is shown by the fact that the maximum variation is only 0.38 per cent, *i. e.*, from 14.38 per cent to 14.76 per cent. This actual difference is very little larger (0.05 per cent) than that observed in the case of sulphur, and when calculated upon the basis of the total amount of sulphur and nitrogen present in the hair the difference between the variation in the nitrogen values is only a little over one-third as great as the variation in the sulphur values. The data for carbon do not show the uniformity observed in the case of nitrogen and sulphur. The values vary from a maximum of 44.34 per cent for the dark brown hair of a girl, seven years of age, to a minimum of 41.65 per cent for the dark brown hair of a girl three years of age. The data for hydrogen also fails to show any particular uniformity. The minimum percentage of hydrogen (6.18) is found in the sample of hair containing the least amount of carbon, *i. e.*, the dark brown hair of a three year old girl; whereas the maximum percentage of hydrogen (6.90) is found in the composite sample of the hair of girls who varied in age from six to nine years.

The ratio, S : N, is rather uniform varying only from 1 :2.8 to

[1] Mohr: *Loc. cit.*

1:3.1. The minimum ratio is possessed by the dark brown hair of a seven year old girl, and the maximum ratio is possessed by the hair of the boys.

The average percentage composition for the whole number of samples of children's hair analyzed is given at the bottom of Table VIII, p. 485. It will be noticed that the average percentage sulphur content (4.93) is the same as that determined for the light hair of girls who were from two and one-half to nine years of age. The average percentage of nitrogen (14.58) is practically duplicated (14.56) by the data from the analysis of the auburn hair of the seven year old girl. The average percentage of carbon (43.23) is in very close agreement with the carbon value obtained from the analysis of the samples of hair obtained from girls from four to twelve years old, whereas the average hydrogen value (6.46 per cent) agrees quite closely with the similar data for the analysis of the light hair of girls two and one-half to nine years of age. The average ratio, S : N (1:3.0), is duplicated by three different samples of hair as will be seen by referring to the tabulated data.

(*d*) *Chemical composition of the hair of caucasian children as influenced by the color of the hair and the sex of the child.* The data for consideration here will be found in tabular form in Table IX, p. 488. An examination of this table will reveal the fact that the variations among the data for the several specimens of hair analyzed are not so great in the case of caucasian children as were noted in the consideration of the hair of the caucasian adults (see Tables VI and VII, pp. 478 and 481), particularly as far as sulphur and nitrogen are concerned. In Table IX we see the maximum percentage of sulphur (5.00) is found in the brown hair of the girls while the minimum percentage (4.77) is found in the hair of the same color obtained from the boys. Of the hair of the females the auburn variety contains the lowest percentage of sulphur (4.80) while the brown hair contains the maximum amount (5.00 per cent) of the element. An examination of the data for the content of nitrogen reveals the fact that the samples of female hair contain amounts of nitrogen which are closely comparable, whereas the specimen of male hair contains rather more of the element. In this connection it is interesting to note that the brown hair of the boys contains the largest

amount of nitrogen (14.76 per cent) as well as the smallest amount (4.77 per cent) of sulphur. The data for the carbon content of the various samples analyzed do not run uniformly with either sulphur or nitrogen but show the maximum percentage (43.87) to be present in the auburn hair of a girl seven years of age and the minimum percentage (42.72) to be present in the light colored hair of girls ranging in age from two and one-half to nine years.

TABLE IX. CHEMICAL COMPOSITION OF THE HAIR OF CAUCASIAN CHILDREN AS INFUENCED BY THE COLOR OF THE HAIR AND THE SEX OF THE CHILD.

Subjects.	Color of Hair.	Average percentage composition of hair.					Ratio S : N	Remarks.
		S	N	C	H	O		
Girl	Auburn	4.80	14.56	43.87	6.26	30.51	1 : 3.0	
Girls	Brown	5.00	14.56	43.12	6.57	30.75	1 : 2.9	
Girls	Light	4.93	14.51	42.72	6.40	31.44	1 : 3.0	Composite sample of 9 specimens.
Girls	(All specimens)	4.96	14.55	43.18	6.49	30.82	1 : 3.0	
Boys	Brown	4.77	14.76	43.56	6.22	30.69	1 : 3.1	Composite sample of 9 specimens.
Average percentage composition of all samples of children's hair.....		4.93	14.58	43.23	6.46	30.80	1 : 3.0	

TABLE X. AVERAGE PERCENTAGE COMPOSITION.

Subject.	Percentage composition of hair.					Ratio S : N.
	S	N	C	H	O	
Indian.....	4.82	15.40	44.06	6.53	29.19	1 : 3.2
Japanese.....	4.96	14.64	42.99	5.91	31.50	1 : 3.0
Negro.....	4.84	14.90	43.85	6.37	30.04	1 : 3.1
Caucasian (adults).....	5.22	15.79	44.49	6.44	28.66	1 : 2.9
Caucasian (children).....	4.93	14.58	43.23	6.46	30.80	1 : 3.0

Hydrogen, on the other hand, resembles sulphur in being present in largest amount (6.57 per cent) in the brown hair of girls and in smallest amount (6.22 per cent) in the brown hair of boys. The ratio, S : N, runs from 1 :2.9 to 1 :3.0 for the hair of the females while the male subjects show the slightly higher ratio, 1 :3.1.

It is interesting to note the close agreement between the results of the analyses of the hair of the caucasian children and the hair

of the japanese. This is shown clearly by a comparison of the data for the japanese hair, as given in Table V, with the average data for the children's hair as given in Tables VIII or IX.

VI. CONCLUSIONS.

The data obtained from the analyses of specimens of hair from the representatives of various races indicate that the chemical composition of the hair is influenced by six factors, as follows:

(1) Race of the individual.
(2) Sex of the individual.
(3) Age of the individual.
(4) Color of the hair.
(5) Purity of breeding of the individual.
(6) Whether the hair sample was obtained from a dead or living person.

CHEMISTRY OF FLESH.

(SIXTH PAPER)[1]

FURTHER STUDIES ON THE APPLICATION OF FOLIN'S CREATIN AND CREATININ METHOD TO MEATS AND MEAT EXTRACTS.

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Since the publication of the first paper[2] from this laboratory upon the determination of creatin and creatinin in meats and meat extracts further studies have been made to test the accuracy of the method formerly used. This additional work has been in part prompted by the paper of Otto Hehner,[3] who takes the attitude that Folin's creatin method if directly applied to commercial meat extracts does not give accurate results. Hehner's chief criticism is that 15 cc. of the 1.2 per cent picric acid solution used in Folin's method as applied to the determination of creatin in urine is not sufficient for the determination of this constituent in commercial meat extracts. In his article Hehner states that in such cases an increased quantity of the picric acid solution should be used and he recommends the use of a total of 25 cc. of a 1.01 per cent solution of picric acid together with "a quite small amount of alkali," and he says further that "an excessive quantity of the alkali diminishes the color." This difference in the quantity and strength of the picric acid which is represented by 6.1 cc. of a 1.2 per cent solution, gave results according to Hehner which showed commercial meat extracts (Lemco, Lazenty's, Armour's

[1] H. S. Grindley: *Journ. of the Amer. Chem. Soc.*, xxvi, p. 1086, 1904; H. S. Grindley and A. D. Emmett: *Ibid.*, xxvii, p. 658, 1905; A. D. Emmett and H. S. Grindley: *Ibid.*, xxviii, p. 25, 1905; P. F. Towbridge and H. S. Grindley: *Ibid.*, xxviii, p. 469, 1906; H. S. Grindley and H. S. Woods: This *Journal* ii, p. 309, 1907.

[2] Grindley and Woods: This *Journal*, ii, p. 309, 1907.

[3] *Pharm. Journ.*, lxxviii, p. 683, 1907.

Baron Liebig's and Army and Navy) to contain 10 to 12 per cent of combined creatin and creatinin, whereas Bauer and Barschall,[1] and Grindley and Woods[2] found by using Folin's proportion of the reagents 4 per cent and 1 to 6.5 per cents, respectively, of these combined extractives. Such differences in the quantities of creatin and creatinin thus obtained in meat extracts by different analysts certainly demand thorough investigation, if this convenient method of determining creatin and creatinin in such preparations is to be retained. In this connection it should be said, that Hehner gives none of his detailed analytical data and the description of his analytical procedure is so meager that it is impossible to accurately decide the details of his method.

Further, as a result of the work already published by Folin[3] and by Benedict and Meyers[4] in testing the method with pure creatin, Hehner's criticism would at first sight seem to be almost without any foundation. On the other hand, the extent to which this method of determining creatin and creatinin is already being used both in commercial and scientific work demands that the conditions under which it gives accurate results should be fully determined experimentally beyond any reasonable doubt. With this object in view, the work here reported was undertaken.

Folin,[5] in first presenting this method for the quantitative determination of creatinin and creatin in urine, recommended the use of 15 cc. of a 1.2 per cent solution of picric acid and 5 cc. of a 10 per cent solution of sodium hydroxide. Later,[6] in modifying his method he increased the amount of alkali from 5 cc. to 9 cc. on account of the increased quantity of normal hydrochloric acid he found desirable to use in changing the creatin to creatinin. Bauer and Barschall,[7] in their work upon beef extracts, used 15 cc. of the picric acid solution and 5 cc. of the alkali solution. Benedict and Meyers,[8] in using the method with urine and with beef

[1] *Arbeiten aus dem kaiserlichen Gesundheitsamte*, xxiv, p. 552.

[2] *Loc. cit.*

[3] *Zeitschr. f. physiol. Chem.*, xli, p. 223, 1904; *Festschrift f. Olaf Hammarsten*, iii, 1906.

[4] *Amer. Journ. of Physiol.*, xviii, p. 4, 1907.

[5] *Zeitschr. f. physiol. Chem.*, xli, p. 223, 1904.

[6] *Festschrift f. Olaf Hammarsten*, iii, 1906.

[7] *Arbeiten aus dem kaiserlichen Gesundheitsamte*, xxiv, p. 552.

[8] *Loc. cit.*

extracts, took 15 cc. of the saturated picric acid solution and 10 cc. of the alkali solution. In the previous publication[1] upon this subject from this laboratory, 15 cc. of the saturated (1.2 per cent) solution of picric acid and 5 cc. of the 10 per cent sodium hydroxide solution were taken. In the work of this laboratory the details of the method as perfected and thoroughly tested by Folin for the determination of creatinin and creatin in urine were followed as closely as possible. Further, in the previous communication, it was clearly stated that the color of the unknown creatinin picrate solution was compared with 8.0 mm. of the $\frac{N}{2}$ solution of potassium bichromate (24.54 grams per liter) and not with the color obtained by allowing the picric acid and alkali to act upon a standard creatinin solution as Hehner states as the method employed by us. In all our work we have accepted the formulas and equivalents, which were so carefully worked out by Folin.

EXPERIMENTAL.

In testing the method, the influence of varying the quantity of both the picric acid solution and the alkali solution was considered. The differences in the intensity of the color, if there be any within narrow limits, due to the length of time the solutions were allowed to stand were not taken up sufficiently in connection with the present study to warrant consideration in this paper. In all the determinations herein reported, the time allowed for the development of the color was as nearly as possible five minutes. The purity and strength of the picric acid solution were thoroughly tested. The picric acid was found to be quite pure and the strength of the picric acid solution used in this work was 1.2 per cent. A 10 per cent solution of sodium hydroxide was used as the alkali. The volumes of the 1.2 per cent solution of the picric acid used were 15, 30 and 45 cc., and 5, 10 and 15 cc. of the 10 per cent solution of the sodium hydroxide were taken. All the readings of the colorimeter were made by at least two persons each working independently of the other. In making the observations each person recorded three or four of those agreeing most closely. One of the analysts in making the read-

[1] This *Journal*, ii, p. 309, 1906.

ings was not informed as to the nature of the solutions under examination. The average of the readings of the two analysts was taken as the true value for the data reported in the tables here given. In cases where the colorimeter was used continuously for a considerable length of time, the standard bichromate solution was renewed once or twice to avoid any possible error due to evaporation.

Detailed description of the method used. About 10 grams of the commercial meat extract were dissolved in water and the resulting solution was diluted to exactly 500 cc. and thoroughly mixed. In some cases the solutions thus prepared were filtered through dry filters while in other cases the original solutions were not filtered before being used for the determination of the creatinin and the creatin. For the determination of the creatinin, aliquot portions of the sample solution which would give a reading of 7 to 9 mm. in the Duboscq colorimeter were transferred to 500 cc. measuring flasks. To these portions, the measured amounts of the 1.2 per cent picric acid solution and the 10 per cent solution of sodium hydroxide were each added. The mixture was then shaken and allowed to stand exactly five minutes when it was immediately and quickly diluted to the 500 cc. mark with distilled water and thoroughly mixed. Readings upon this solution were made at once in the colorimeter, comparing the depth of color of the solution to be tested with that of a standard half-normal bichromate (24.54 grams per liter) solution set at 8 mm.

For the determination of the creatin measured portions of the sample solution were transferred to small Jena beakers. In case the volumes of the solution taken amounted to more than 10 cc., they were evaporated upon the steam bath to this volume and then treated with 10 cc. of normal hydrochloric acid. The creatin of the solutions was changed to creatinin by the autoclave method of Benedict and Meyers[1] which has proved to be convenient and accurate. To do this, the beakers containing the solutions were placed in an autoclave and heated at a temperature of 117° C.–120° C. for 30 minutes. After these solutions were taken from the autoclave they were diluted to a definite volume

[1] *Amer. Journ. of Physiol.*, xviii, p. 398, 1907.

and measured amounts of the diluted solutions which would give a reading of 7 to 9 mm. in the colorimeter were transferred to 500 cc. measuring flasks. The details of procedure were then continued exactly as directed above for the estimation of the creatinin. The data of the experiments are given below in full.

Experiment 1. For this experiment 9.2899 grams of a well-known brand of commercial beef extract were dissolved in water and the resulting solution was diluted to 500 cc. and thoroughly mixed. The solution was then filtered through dry filters and portions of 25 cc. each were taken for the determination of creatinin by the method described in detail above. The results of the determination, in which varying amounts of the picric acid and the sodium hydroxide solutions were used for the same volume, of the sample solutions are given in the following table:

TABLE 1. CREATININ RESULTS OBTAINED IN EXPERIMENT 1.

Lab. No.	SAMPLE.	METHOD.		Reading of colorimeter.	Weight of creatinin.	Total weight of creatinin.	Per cent of creatinin.
		Picric acid used.	Alkali used.				
		cc.	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*
2278a	Beef extract.....	15	5	9.0	9.000	180.00	1.94
2278a	" "	15	10	8.9	9.101	182.02	1.96
2278a	" "	30	5	8.5	9.529	190.58	2.05
2278a	" "	30	5	8.9	9.101	182.02	1.96
2278a	" "	30	10	8.4	9.643	192.86	2.08
2278a	" "	30	10	8.2	9.878	197.56	2.13
2278a	" "	45	5	9.0	9.000	180.00	1.94
2278a	" "	45	10	8.9	9.101	182.02	1.96
	Average						*2.00*

Experiment 2. For this experiment the same filtered solution of beef extract was taken for the determination of creatin as was used above in Experiment 1 for the determination of creatinin. For the estimation of the creatin in the solution, five portions of 25 cc. each of the original filtered solution were measured out and the creatin which they contained was converted into creatinin in the autoclave as described above. The five solutions representing 125 cc. of the original solution of the extract after removal from the autoclave were united and diluted to 500 cc. Fifty cubic centimeter portions of this diluted solution were taken for the determination of creatinin by the method described in full above. The analytical results obtained, where varying amounts of the picric acid solution and the sodium hydroxide solution were used for the same volume of the sample solution, are given in the following table:

TABLE 2. CREATIN RESULTS OBTAINED IN EXPERIMENT 2.

Lab. No.	SAMPLE.	METHOD.		Reading of colorimeter.	ORIGINAL CREATININ PLUS CREATININ DUE TO CREATIN.			Per cent of original creatinin.	Per cent of creatinin due to creatin.	Per cent of creatin (creatinin due to creatin × 1.16).
		Picric acid used.	Alkali used.		Original weight.	Total weight.	Per cent.			
		cc.	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*	*p. ct.*	*p. ct.*	*p. ct.*
2278a	Beef extract.....	15	5	16.1	5.032	201.28	2.17	2.00	0.17	0.20
2278a	" "	15	10	10.5	7.714	308.56	3.32	2.00	1.32	1.53
2278a	" "	15	15	9.6	8.438	337.52	3.63	2.00	1.63	1.89
2278a	" "	30	10	9.0	9.000	360.00	3.88	2.00	1.88	2.18
2278a	" "	30	10	9.1	8.901	356.04	3.83	2.00	1.83	2.12
2278a	" "	30	15	9.0	9.000	360.00	3.88	2.00	1.88	2.18
2278a	" "	30	15	9.2	8.804	352.16	3.79	2.00	1.79	2.08
2278a	" "	45	10	8.9	9.101	364.04	3.92	2.00	1.92	2.23
2278a	" "	45	10	9.0	9.000	360.00	3.88	2.00	1.88	2.18

Experiment 3. For this experiment two solutions were prepared from the same beef extract as that used in Experiments Nos. 1 and 2. In the first, namely, No. 2278b, 11.3131 grams of the extract were dissolved in water and the resulting solution was diluted to 500 cc. and thoroughly mixed. In the second solution, No. 2278c, 10.3424 grams of the extract were dissolved in water and made up to a definite volume of 500 cc. These solutions were not filtered but they were used directly for the work reported in this and the following experiment. Portions of 25 cc. each of these unfiltered solutions were taken for the determination of creatinin.

The results of the tests are given in the following table:

TABLE 3. CREATININ RESULTS OBTAINED IN EXPERIMENT 3.

Lab. No.	Sample.	Method. Picric acid used.	Method. Alkali used.	Reading of colorimeter.	Weight of creatinin.	Total weight of creatinin.	Per cent of creatinin.
		cc.	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*
2278b	Beef extract.....	15	5	7.1	11.408	228.16	2.02
2278b	" "	30	5	6.9	11.739	234.78	2.08
2278b	" "	30	10	6.9	11.739	234.78	2.08
2278b	" "	45	5	6.9	11.739	234.78	2.08
2278b	" "	45	10	6.9	11.739	234.78	2.08
	Average.....						*2.07*
2278c	Beef extract.....	15	5	7.8	10.385	207.70	2.01
2278c	" "	15	5	7.8	10.385	207.70	2.01
2278c	" "	15	10	7.8	10.385	207.70	2.01
2278c	" "	15	10	7.9	10.253	205.06	1.98
2278c	" "	21	10	7.8	10.385	207.70	2.01
2278c	" "	21	10	7.8	10.385	207.70	2.01
2278c	" "	21	15	7.8	10.385	207.70	2.01
2278c	" "	21	15	7.8	10.385	207.70	2.01
2278c	" "	30	10	7.8	10.385	207.70	2.01
2278c	" "	30	10	7.8	10.385	207.70	2.01
	Average.....						*2.01*

Experiment 4. For this experiment the same unfiltered solution, No. 2278c, was taken for the determination of creatin as was used above for the determination of creatinin in Experiment 3. For the estimation of the creatin four portions of 25 cc. each of the original unfiltered solution were measured out and the creatin which they contained was changed into creatinin as usual. The four solutions representing 100 cc. of the original solution of the extract after removal from the autoclave were united and diluted to 200 cc. Twenty-five cubic centimeter portions of this diluted solution were taken for the determination of creatinin by the usual method. The results of the tests are given in the following table:

TABLE 4. CREATIN RESULTS OBTAINED IN EXPERIMENT 4.

Lab. No.	SAMPLE.	METHOD.		Reading of colorimeter.	ORIGINAL CREATININ PLUS CREATININ DUE TO CREATIN.			Per cent of original creatinin.	Per cent of creatinin due to creatin.	Per cent of creatin (creatinin due to creatin × 1.16).
		Picric acid used.	Alkali used.		Original weight.	Total weight.	Per cent.			
		cc.	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*	*p. ct.*	*p. ct.*	*p. ct.*
2278b	Beef extract.....	15	10	8.7	9.310	372.40	3.60	2.01	1.59	1.84
2278b	" " ...	15	10	8.8	9.205	368.20	3.56	2.01	1.55	1.80
2278b	" " ...	21	10	8.7	9.310	372.40	3.60	2.01	1.59	1.84
2278b	" " ...	21	10	8.8	9.205	368.20	3.56	2.01	1.55	1.80
2278b	" " ...	30	10	8.2	9.878	395.12	3.82	2.01	1.81	2.10
2278b	" " ...	30	10	8.2	9.878	395.12	3.82	2.01	1.81	2.10

Experiment 5. For this experiment a cold water extract of 150.5287 grams of lean beef round was prepared by the methods described in a former paper.[1] The resulting extract was made up to a volume of 7500 cc. Five portions of 1000 cc. each of this cold water extract were measured out and evaporated to a volume of about 50 cc. The coagulated proteid resulting was removed by filtration and thoroughly washed with hot water. The filtrates were now evaporated to a volume of 10 cc. and the creatin[2] which they contained was converted into creatinin by use of the autoclave. The five solutions representing five liters of the original cold water extract after removal from the autoclave were united and diluted to 500 cc. Fifteen cubic centimeter portions of this last solution were taken for the determination of creatinin by the usual method. The results of the tests are given in the following table:

TABLE 5. CREATIN RESULTS OBTAINED IN EXPERIMENT 5.

Lab. No.	SAMPLE.	METHOD.		Reading of colorimeter.	Weight of creatinin due to creatin.	Total weight of creatinin (A).	Total weight of creatin. (A × 1.16.)	Per cent of creatin.
		Picric acid used.	Alkali used.					
		cc.	*cc.*	*mm.*	*mgr.*	*mgr.*	*mgr.*	*p. ct.*
2279	Lean beef round.....	15	5	7.3	11.096	554.80	643.57	0.428
2279	" "	15	5	7.7	10.519	525.95	610.10	0.405
2279	" "	15	5	7.4	10.950	547.50	635.10	0.422
2279	" "	15	5	7.5	10.800	540.00	626.40	0.416
2279	" "	15	5†	7.7	10.520	526.00	610.16	0.405
2279	" "	15	10	7.5	10.800	540.00	626.40	0.416
2279	" "	15	10*	7.8	10.380	519.00	602.04	0.400
2279	" "	30	10	7.0	11.570	578.50	671.06	0.446
2279	" "	30	10	7.0	11.570	578.50	671.06	0.446
2279	" "	30	10†	7.2	11.250	562.50	652.50	0.434
2279	" "	30	15	7.0	11.570	578.50	671.06	0.446
2279	" "	30	10	7.0	11.570	578.50	671.06	0.446
2279	" "	45	15	7.0	11.570	578.50	671.06	0.446

* Also added 10 cc. $\frac{N}{1}$ HCl.
† Also added 10 cc. NaCl, 6 per cent.

[1] H. S. Grindley and A. D. Emmett: *Journ. of the Amer. Chem. Soc.*, xxvii, p. 658, 1905.

[2] Former experiments made in this laboratory demonstrated the fact that fresh meats contain only the slightest trace of creatinin if any at all.

Experiment 6. For this experiment five different brands of beef extracts were taken. They were given Laboratory Nos. 2306, 2307, 2308, 2309 and 2310. The following weights of these extracts were respectively taken for the work, 11.7990, 10.1120, 9.5615, 11.2308, and 9.6105 grams. Each of these weighed portions was dissolved in water and the solutions thus obtained were diluted to 500 cc. and thoroughly mixed. The resulting solutions were not filtered but they were used directly for the work reported in this and the following experiment. The portions of these diluted solutions which were taken for the several determinations are given below in the table giving the results of the tests of this experiment.

TABLE 6. CREATININ RESULTS OBTAINED IN EXPERIMENT 6.

Lab. No.	SAMPLE.	Original volume taken.	METHOD. Picric acid used.	METHOD. Alkali used.	Reading of colorimeter.	Weight of creatinin.	Total weight of creatinin.	Per cent of creatinin.
		cc.	*cc.*	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*
2306	Beef extract.....	8.5	15	5	7.5	10.80	635.25	5.38
2306	" "	8.5	15	10	7.4	10.95	644.08	5.47
2306	" "	8.5	30	10	7.2	11.25	661.73	5.61
2306	" "	8.5	30	10	7.3	11.09	652.31	5.53
	Average							*5.45*
2307	Beef extract.....	23.0	15	5	8.0	10.13	220.26	2.18
2307	" "	23.0	15	10	7.8	10.38	225.66	2.23
2307	" "	23.0	30	10	7.6	10.66	231.75	2.29
	Average							*2.23*
2308	Beef extract.....	50.0	15	10	7.8	10.39	103.85	1.09
2308	" "	50.0	21	10	7.8	10.39	103.85	1.09
2308	" "	50.0	21	10	7.8	10.39	103.85	1.09
2308	" "	50.0	30	10	7.8	10.39	103.85	1.09
2308	" "	50.0	30	10	7.8	10.39	103.85	1.09
	Average							*1.09*
2309	Beef extract.....	13.0	15	5	7.3	11.096	426.75	3.80
2309	" "	13.0	15	5	7.3	11.096	426.75	3.80
2309	" "	13.0	15	10	7.2	11.225	432.68	3.85
2309	" "	13.0	15	10	7.0	11.571	445.02	3.96
2309	" "	13.0	30	10	7.0	11.571	445.02	3.96
2309	" "	12.5	30	10	7.3	11.096	443.84	3.95
2309	" "	13.0	30	10	7.1	11.408	438.75	3.91
2309	" "	13.0	30	10	7.1	11.408	438.75	3.91
2309	" "	13.0	30	15	7.1	11.408	438.75	3.91
	Average							*3.89*
2310	Beef extract.....	17.0	15	5	7.0	11.57	340.27	3.54
2310	" "	17.0	15	10	7.0	11.57	340.27	3.54
2310	" "	17.0	30	10	6.9	11.74	344.09	3.58
	Average							*3.55*

Experiment 7. For this experiment the same unfiltered solutions of the five beef extracts used above in Experiment 6 were taken for the determination of creatin. For the estimation of creatin Nos. 2306, 2307, 2309 and 2310, five portions of 25 cc. each were measured from each of the four solutions of the extracts and the creatin which they contained was converted into creatinin as usual. The five solutions representing 125 cc. of the original solutions of each of the extracts after removal from the autoclave were united and diluted to 500 cc. For the estimation of creatin in No. 2308 four portions of 25 cc. each were measured from the original solution of this extract and the creatin contained in the same was converted into creatinin as usual. The four solutions representing 100 cc. of the original solution after removal from the autoclave were united and diluted to 200 cc. The portions of these diluted solutions which were taken for the determination of the creatinin by the usual method are indicated by the following table which gives the detailed analytical results for the three samples of beef extract.

TABLE 7. CREATIN RESULTS OBTAINED IN EXPERIMENT 7.

Lab. No.	Sample.	Diluted sol. taken.	Method.		Reading of colorimeter.	Original creatinin plus creatinin due to creatin.			Per cent of original creatinin.	Per cent of creatinin due to creatin.	Per cent of creatin (creatinin due to creatin × 1.16).
			Picric acid used.	Alkali used.		Original weight.	Total weight.	Per cent.			
		cc.	cc.	cc.	mm.	mgr.	mgr.	p. ct.	p. ct.	p. ct.	p. ct.
2306	Beef extract..	50	15	5	7.0	11.571	462.84	3.92	5.45		
2306	" "	50	15	5	7.1	11.408	456.32	3.87	5.45		
2306	" "	30	30	10	7.0	11.571	771.79	6.54	5.45	1.09	1.26
2307	" "	50	15	5	11.0	7.364	294.56	2.91	2.23	0.68	0.79
2307	" "	70	15	10	6.9	11.594	331.23	3.28	2.23	1.05	1.22
2307	" "	70	30	10	6.4	12.656	361.58	3.58	2.23	1.35	1.57
2308	" "	60	30	10	9.0	9.000	150.00	1.57	1.09	0.48	0.57
2308	" "	67	30	10	8.3	9.759	145.70	1.52	1.09	0.43	0.50
2309	" "	37	15	5	9.1	8.901	481.10	4.28	3.89	0.39	0.45
2309	" "	37	15	5	9.0	9.000	486.45	4.33	3.89	0.44	0.51
2309	" "	37	15	5	8.9	9.101	491.91	4.38	3.89	0.49	0.57
2309	" "	37	15	10	8.0	10.125	547.26	4.87	3.89	0.98	1.14
2309	" "	37	15	10	7.9	10.253	554.18	4.93	3.89	1.04	1.21
2309	" "	37	15	10	7.8	10.385	561.31	5.00	3.89	1.11	1.29
2309	" "	37	30	10	7.7	10.519	568.55	5.06	3.89	1.17	1.36
2309	" "	37	30	15	7.9	10.253	554.18	4.93	3.89	1.04	1.21
2310	" "	50	15	5	8.1	10.000	400.00	4.16	3.54	0.62	0.72
2310	" "	50	15	10	5.8	13.966	558.64	5.81	3.54	2.27	2.63
2310	" "	50	30	10	5.6	14.464	578.56	6.02	3.54	2.48	2.88
2310	" "	35	30	10	7.9	10.253	585.45	6.09	3.54	2.55	2.96

Experiment 8. For this experiment three samples of urine from three different persons were taken. These samples were given Laboratory Nos. 2319, 2320 and 2321. The specific gravities of the urines were respectively, 1.033, 1.029, 1.018. In each sample the creatinin was determined by the usual method, using however different quantities of picric acid and varying quantities of alkali. The detailed results of the experiment are given in the table that follows:

TABLE 8. CREATININ RESULTS OBTAINED IN EXPERIMENT 8.

Lab. No.	SAMPLE.	Original volume taken.	METHOD. Picric acid used.	METHOD. Alkali used.	Reading of colorimeter.	Weight of creatinin.	Total weight of creatinin	Per cent of creatinin.
		cc.	*cc.*	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*
2319	Urine.....	4.0	15	5	6.9	11.740	205.45	0.28
2319	"	4.0	15	5	7.0	11.570	202.48	0.28
2319	"	4.0	15	10	6.9	11.740	205.45	0.28
2319	"	4.0	30	10	6.4	12.660	221.55	0.31
2319	"	4.0	30	10	6.5	12.460	218.05	0.30
	Average							*0.29*
2320	Urine.....	4.3	15	5	7.8	10.380	142.41	0.23
2320	"	4.3	30	10	7.4	10.950	150.23	0.25
	Average							*0.24*
2321	Urine.....	6.5	15	5	7.4	10.950	176.78	0.17
2321	"	6.5	15	10	7.3	11.100	179.20	0.17
2321	"	6.5	15	10	7.5	10.800	174.42	0.17
2321	"	6.5	30	10	7.3	11.100	179.20	0.17
2321	"	6.5	30	15	7.3	11.100	179.20	0.17
	Average							*0.17*

Experiment 9. The same samples of urine as were used above in Experiment 8 were taken for the determination of creatin. For the estimation of the creatin in Laboratory Nos. 2319 and 2321 four portions of 10 cc. each were measured from each sample, and the creatin which they contained was changed into creatinin as usual. The four solutions of each sample representing 40 cc. of the original urine after removal from the autoclave were united and diluted to 250 cc. The portions of the diluted solution which were taken for the determination of the creatinin are indicated in the table. For the estimation of creatin in Laboratory No. 2320 three portions of 10 cc. each were taken for changing the creatin into creatinin. The three solutions representing 30 cc. of the original urine after removal from the autoclave were united and diluted to 250 cc. The portions taken for the tests are indicated in the table. The complete analytical data for the creatin determination in the three samples of urine are given in detail in the table below:

TABLE 9. CREATIN RESULTS OBTAINED IN EXPERIMENT 9.

Lab. No.	Sample.	Diluted solution taken.	Method.		Reading of colorimeter.	Original creatinin plus creatinin due to creatin.			Per cent of original creatinin.
			Picric acid used.	Alkali used.		Original weight.	Total weight.	Per cent.	
		cc.	*cc.*	*cc.*	*mm.*	*mgr.*	*mgr.*	*p. ct.*	*p. ct.*
2319	Urine	20.0	15	5	8.4	9.643	210.94	0.29	0.28
2319	"	20.0	15	10	8.2	9.878	216.08	0.30	0.28
2319	"	20.0	15	10	8.2	9.878	216.08	0.30	0.28
2319	"	20.0	30	10	8.1	10.000	218.75	0.30	0.30
2319	"	25.0	30	10	6.4	12.656	221.48	0.31	0.31
2319	"	20.0	30	15	8.3	9.759	213.48	0.30	....
2320	"	31.5	15	5	8.8	9.205	143.69	0.24	0.24
2320	"	31.5	15	10	8.7	9.310	145.33	0.24	0.24
2320	"	31.5	15	10	8.8	9.205	143.69	0.24	0.24
2320	"	25.0	30	10	10.0	8.100	159.33	0.26	0.24
2320	"	31.5	30	10	8.5	9.529	148.75	0.25	0.24
2320	"	31.5	30	10	8.5	9.529	148.75	0.25	0.24
2321	"	34.5	15	10	8.8	9.205	175.08	0.16	0.17
2321	"	34.5	15	10	9.0	9.000	171.18	0.16	0.17
2321	"	34.5	30	10	8.8	9.205	175.08	0.16	0.17
2321	"	34.5	30	10	9.0	9.000	171.18	0.16	0.17
2321	"	25.0	30	10	10.9	7.431	195.06	0.18	0.17

Experiment 10. As a further check on this method, we were fortunate enough to obtain through the kindness of Dr. Folin some pure creatin (Kahlbaum). Crystallized creatin contains one molecule of water of crystallization and in our sample the moisture content was ascertained and duly allowed for in the subsequent data in Table 10. Several portions of the sample, given Laboratory No. 2289, were weighed off and each dissolved in an appropriate quantity of distilled water. The following weights of the dry substance were taken:

(*a*) .1290 grams	(*b*) .2217 grams	(*c*) .1165 grams
(*d*) .2705 "	(*e*) .2217 "	(*f*) .4699 "

All of the samples were dissolved in 100 cc. of distilled water, except in *f*, which was dissolved in 400 cc. From these solutions, five 10 cc. portions of samples, *b*, *d*, *e* and *f*, and nine 10 cc. portions of samples *a* and *c* were transferred to small beakers. To each beaker 10 cc. of normal hydrochloric acid was added, after which the dehydration was carried on at 117–119° C. in the autoclave for one-half hour. Each of the resulting creatinin solutions of the respective samples was transferred to a 500 cc. measuring flask and diluted to the mark. After thoroughly mixing, portions of 50 cc. were taken for the usual colorimetric determinations. Special mention should perhaps be made at this point for subsequent discussion that solutions *a* and *c* contained the equivalent of 90 cc. of normal hydrochloric acid and that the others, *b*, *d*, *e* and *f*, contained 50 cc.

The following table gives the results of the several tests:

TABLE 10. SUMMARY OF RESULTS OBTAINED IN EXPERIMENT 10.

Lab. No.	Weight of dry sample.	Original volume.	Diluted volume taken.	METHOD. Picric acid.	METHOD. Alkali.	Reading of colorimeter.	Weight of creatinin due to creatin.	Total weight of creatinin due to creatin.	Weight of creatin (Creatinin × 1.16).	Per cent of creatin.
	gms.	*cc.*	*cc.*	*cc.*	*cc.*	*mm.*	*mgr.*	*mgr.*	*mgr.*	*p. ct.*
2289A	0.1290	100	50	15	5	22.6	3.580	39.82	46.19	35.81
2289A	0.1290	100	50	15	5	22.6	3.580	39.82	46.19	35.81
		Average (2)				*22.6*	*3.580*	*39.82*	*46.19*	*35.81*
2289A	0.1290	100	50	15	10	9.1	8.901	98.89	114.72	88.93
2289A	0.1290	100	50	15	10	9.1	8.901	98.89	114.72	88.93
		Average (2)				*9.1*	*8.901*	*98.89*	*114.72*	*88.93*
2289A	0.1290	100	50	30	10	8.4	9.643	107.14	124.29	96.34
2289A	0.1290	100	50	30	10	8.5	9.529	105.88	122.82	95.21
		Average (2)				*8.5*	*9.586*	*106.51*	*123.56*	*95.78*
2289A	0.1290	100	50	30	15	8.5	9.529	105.88	122.82	95.21
2289B	0.2217	100	50	15	10	9.5	8.526	170.52	197.80	89.21
2289B	0.2217	100	50	15	10	9.4	8.617	172.34	199.91	90.17
		Average (2)				*9.5*	*8.572*	*171.43*	*198.86*	*89.69*

Lab. No.	Weight of dry sample.	Original volume.	Diluted volume taken.	METHOD. Picric acid.	METHOD. Alkali.	Reading of colorimeter.	Weight of creatinin due to creatin.	Total weight of creatinin due to creatin.	Weight of creatin (Creatinin × 1.16).	Per cent of creatin.
2289B	0.2217	100	50	30	10	9.0	9.000	180.00	208.80	94.18
2289B	0.2217	100	50	30	15	9.0	9.000	180.00	208.80	94.18
2289C	0.1165	100	63	15	10	9.0	9.000	89.29	103.58	88.91
2289C	0.1165	100	63	15	10	8.8	9.110	90.37	104.83	89.99
		Average (2)				*8.9*	*9.055*	*89.83*	*104.21*	*89.45*
2289C	0.1165	100	63	30	10	8.5	9.529	94.54	109.66	94.13
2289C	0.1165	100	63	30	10	8.4	9.643	95.67	110.97	95.25
		Average (2)				*8.5*	*9.586*	*95.11*	*110.32*	*94.69*
2289C	0.1165	100	63	30	15	8.5	9.529	94.54	109.66	94.13
2289D	0.2705	100	50	15	5	9.0	9.000	180.00	208.80	76.89
2289D	0.2705	100	50	15	10	7.7	10.519	210.38	244.04	90.21
2289D	0.2705	100	50	15	10	7.6	10.658	213.16	247.27	91.04
		Average (2)				*7.7*	*10.589*	*211.77*	*245.65*	*90.63*
2289D	0.2705	100	50	30	10	7.2	11.250	225.10	261.11	96.49
2289D	0.2705	100	50	30	10	7.3	11.096	221.92	257.43	95.17
2289D	0.2705	100	50	30	10	7.3	11.096	221.92	257.43	95.17
2289D	0.2705	100	50	30	10	7.4	10.946	218.92	253.95	93.88
		Average (4)				*7.3*	*11.097*	*221.97*	*257.48*	*95.18*
2289D	0.2705	100	50	30	15	7.4	10.946	218.92	253.95	93.88
2289E	0.2217	100	50	15	5	11.1	7.300	145.94	169.29	76.36
2289E	0.2217	100	50	15	10	9.0	9.000	180.00	208.80	94.18
2289E	0.2217	100	50	15	10	8.9	9.101	182.02	211.14	95.28
		Average (2)				*9.0*	*9.051*	*181.01*	*209.97*	*94.71*
2289E	0.2217	100	50	30	10	8.9	9.101	182.02	211.14	95.24
2289E	0.2217	100	50	30	10	8.9	9.101	182.02	211.14	95.24
2289E	0.2217	100	50	30	10	8.9	9.101	182.02	211.14	95.24
		Average (3)				*8.9*	*9.101*	*182.02*	*211.14*	*95.24*
2289F	0.4699	200	50	15	5	10.2	7.940	317.64	368.46	78.47
2289F	0.4699	200	50	15	5	10.4	7.788	311.52	361.36	76.88
		Average (2)				*10.3*	*7.864*	*314.58*	*364.91*	*77.68*
2289F	0.4699	200	50	15	10	8.4	9.643	385.72	447.44	95.22
2289F	0.4699	200	50	15	10	8.3	9.759	390.36	452.82	96.38
		Average (2)				*8.4*	*9.701*	*388.04*	*450.13*	*95.80*
2289F	0.4699	200	50	30	10	8.0	10.125	405.00	469.80	99.98
2289F	0.4699	200	50	30	10	8.0	10.125	405.00	469.80	99.98
		Average (2)				*8.0*	*10.125*	*405.00*	*469.80*	*99.98*
2289F	0.4699	200	50	30	15	8.0	10.125	405.00	469.80	99.98

Experiment 11. For this experiment several tests were made upon solutions of creatinin (Merck's). This product was found to be only about 85 per cent pure, there being also present approximately 10 per cent of creatin and 2.07 per cent of moisture. As a result, the material in question could not be used to serve the double purpose intended, namely, the checking of the method and the standardizing of the bichromate solution.

However, to ascertain the variations, if any, in the percentage of creatinin in the sample when different proportions of the picric acid and alkali were used three portions were weighed out. Sample *a* weighed 0.1286 grams (dry); *b*, 0.1127 grams (dry), and *c*, 0.2055 grams (dry). The first two samples were dissolved in 500 cc. of distilled water and after thoroughly mixing, 50 cc. were taken for the usual procedure. Sample *c* was dissolved in 250 cc. of water and 100 cc. of this solution were taken and diluted to 500 cc. after which 75 cc. were used for the regular determination.

The detailed data resulting are given in the following table:

TABLE 11. SUMMARY OF RESULTS OBTAINED IN EXPERIMENT 11.

Lab. No.	Weight of sample.	Original volume.	Original or diluted volume taken.	METHOD.		Reading of colorimeter.	Weight of Creatinin.	Total weight of creatinin.
				Picric acid	Alkali. 1			
	gms.	*cc.*	*cc.*	*cc.*	*cc.*	*mm.*	*mgr.*	*mgr.*
2353a	.1286	500	50	15	5	8.7	9.310	93.10
2353a	.1286	500	50	15	5	8.8	9.205	92.05
2353a	.1286	500	50	15	10	7.6	10.658	106.58
2353a	.1286	500	50	15	10	7.6	10.658	106.58
2353a	.1286	500	50	30	10	7.4	10.946	109.46
2353a	.1286	500	50	30	10	7.5	10.800	108.00
2353a	.1286	500	50	30	15	7.4	10.946	109.46
2353a	.1286	500	50	30	15	7.5	10.800	108.00
2353b	.1127	500	50	15	5	10.2	7.941	79.41
2353b	.1127	500	50	15	10	8.8	9.205	92.05
2353b	.1127	500	50	15	10	8.8	9.205	92.05
2353b	.1127	500	50	30	10	8.6	9.415	94.15
2353b	.1127	500	50	30	10	8.7	9.310	93.10
2353b	.1127	500	50	30	15	8.8	9.205	92.05
2353c	.2055	250	75	15	10	9.0	9.000	150.03
2353c	.2055	250	75	15	10	9.0	9.000	150.03
2353c	.2055	250	75	30	10	8.0	10.125	168.78
2353c	.2055	250	75	30	10	8.0	10.125	168.78
2353c	.2055	250	75	30	15	8.0	10.125	168.78

DISCUSSION OF RESULTS.

It is quite apparent from the data reported in the preceding pages that the Folin method of estimating creatinin and creatin, when properly modified, is as applicable to meat and meat extract as it is to urine. Further, it is evident that if the details of the method are properly followed, reliable and concordant results can be obtained.

ORIGINAL CREATININ. (*a*) *Influence of alkali.* By comparing the data in Tables 1, 3, 6 and 8 which relate to the percentage of creatinin in meat extracts and urine, it is seen that in so far as the initial quantity of creatinin is concerned, the influence of increasing the amount of alkali is practically nil. If 15, 21 or 30 cc. of picric acid of 1.2 per cent are used with either 5, 10 or 15 cc. of sodium hydroxide of 10 per cent strength the readings of the colorimeter for the *same* volume of the solutions under examination, are in general slightly higher for the 5 cc. quantities than for the 10 cc. portions. Stating these facts in terms of percentages of creatinin, the 10 and 15 cc. quantities of sodium hydroxide, which are approximately 8 and 13 cc., respectively, in excess of the necessary amount to cause neutralization and solution of the precipitate, gave but very slightly higher percentages than did the 5 cc. quantities. These actual differences in the case of meat extracts were 0.02 to 0.17 per cent, being on the average 0.05 per cent. In the case of the creatinin (Merck's), which as previously stated contained 10 per cent of creatin, the data in Table 11, show the variations in amount of alkali to produce an appreciable difference. The weights of original creatinin resulting from the use of 10 or 15 cc. of alkali are the same, but for those resulting from the use of 5 cc., they are distinctly lower, being in the former cases on an average of 106.58 mgms., and in the latter 92.58 mgms., a difference of 14.0 mgms. No positive explanation can be given for this large variation. It is easily seen that the differences in some cases in the readings due to the amounts of alkali might be from 2 to 4 mm. as was the case with the beef extracts and urine, and this would mean with such a strong solution, a difference of 2 to 4 per cent.

From the above discussion, the data show that, contrary to Hehner's statement, an excess of alkali, using 5, 10 and 15 cc. of

a 10 per cent solution, does not diminish the depth of the color produced with creatinin picrate but rather increases it. It would seem, therefore, that it would be best to use 10 cc. of a 10 per cent solution of alkali in all instances, it having been shown that this amount of alkali has no detrimental effect whatever.

(*b*) *Influence of picric acid.* In studying the data in Tables 1, 3 and 6 on beef extract, 8 on urine, and 11 on pure creatinin, it will be seen that the variations in the percentages of original creatinin are extremely slight when the quantity of picric acid is increased from 15 to 21, 30 or 45 cc. in the cases where 10 cc. of the alkali are used. In fact, it can be stated, as far as the creatinin sample and also the meat extract samples are concerned, that the 15 cc. test of picric acid (1.2 per cent) gave as high a percentage of creatinin as did the 21 cc. test (which is the equivalent of Hehner's 25 cc. portion, 1.01 per cent), or even the 30 cc. test. This fact is most clearly brought out in Tables 3 and 6 where the analysis of six different samples of meat extract are reported. Sample 2306 has a variation, taking the average of the readings of the 30 cc. tests, of 0.10 per cent on a total of 5.47 per cent of original creatinin. Sample 2307 shows an increase of 0.06 per cent for the 30 cc. of the picric acid against that of 15 cc. on a total of 2.23 per cent. Similarly, the data for sample 2310 gave a gain of 0.04 per cent for the 30 cc. of picric acid when compared with the 15 cc. on a percentage of creatinin of 3.55. These slight differences are no greater than the errors which might be due to the matching of the color, to the carrying out of the technique of the method or to the sampling of the products.

In the case of the urine, Table 8, the variations in the readings for sample 2319 are greater than in the cases just considered but those for sample 2321 show the same variations as the meat extracts. When calculated to the percentage of creatinin these differences in the urine are insignificant. Further, when the analysis of the three samples of the pure creatinin are considered, it is seen that the readings of the two tests, 15 and 30 cc. of picric acid are almost identical. These small differences are no greater than would be expected. However, if these values are calculated to percentages, the variations are about 1.0 per cent. The cause of this difference is not to be found as Hehner

states in the greater amount of creatinin in the solution which is being treated with picric acid, because in this, and the previous work of this laboratory, whether the sample under examination was meat extract, urine, or creatinin itself, such an aliquot portion of the *original* solution was taken that when it was treated in the usual manner with picric acid and alkali, and diluted, the resulting color gave a reading of 7 to 9 mm. on the scale of the colorimeter when compared with the standard bichromate set at 8 mm. In other words, all the solutions tested at the time of the reading contained approximately the same quantity of original or converted creatinin. The real cause of the influence of the slight differences in the readings upon the variations calculated in percentage, naturally lies in the differences in the weights of the substances taken for the samples, since the proportion of the dilutions were approximately the same. The weights of the samples were always about 150 grams for meats, 10 grams for meat extracts and 0.12 grams for the creatinin, and, consequently, it is plainly seen that any variation in the readings would be very much less apparent in the percentage composition in the case of the meats than in the meat extract or the impure creatinin.

From this discussion upon the influence of varying the quantity of picric acid in cases where 10 cc. of a 10 per cent solution of alkali were used, it seems safe to state that it makes no difference, when determining the original creatinin, whether 15, 21 or 30 cc. of the 1.2 per cent solution of picric acid are used. The final percentages are practically identical.

CREATININ DUE TO CREATIN. (*a*) *Influence of alkali.* In the former paper, it was stated that the creatin in the samples was changed to creatinin by using 25 cc. of $\frac{N}{10}$ hydrochloric acid and that the resulting solutions after proper dilution were treated with 15 cc. of picric acid (1.2 per cent) and 5 cc. of alkali (10 per cent). The data herein reported were ascertained by using 10 cc. of normal hydrochloric acid for the dehydration or four times the amount previously taken. Following this change in the amount of acid, the quantity of alkali was also increased. These modifications were adopted on account of the recommendations of Folin in his second paper,[1] that in the determining of creatin in

[1] *Festschrift f. Olaf Hammarsten*, iii, 1906.

abnormal urine 10 cc. of normal hydrochloric acid, 15 cc. of picric acid (1.2 per cent) and 9 cc. of sodium hydroxide (10 per cent) should be taken.

In studying the influence of different proportions of alkali when varying amounts of dehydrated creatin and original creatinin were present, two series of tests were carried out. In the first, 5 and 10 cc. of the alkali were used against 15 cc. of the picric acid, and in the second, 10 and 15 cc. of the alkali were employed with 30 and sometimes 45 cc. of the picric acid. It was ascertained in the case of meat extracts that approximately 1 to 2 cc. of the 10 per cent alkali were sufficient to dissolve the precipitate and to produce a red coloration.

The data in Tables 2, 4 and 7, relating to meat extracts, show the effect of using 5 and 10 cc. of the alkali with 15 cc. of the picric acid. It is very apparent that the 5 cc. portion is entirely too small. This fact is perhaps shown most markedly in sample 2306, where the total creatinin is 3.90 per cent while that for the original creatinin is 5.45 per cent. In other words, the 5 cc. of alkali produced a color which, when compared with the standard, represented a value of 29.4 per cent less than the amount obtained before dehydration. A comparison of the 5 and 10 cc. tests is best brought out in the case of samples 2278a, Table 2; and 2309 and 2310, Table 7. The differences in the readings with the two quantities of alkali vary from 1.1 mm. in 2309 to 5.6 mm. in 2278a. These data when calculated to the percentage of creatinin as creatin, show a range of 0.7 to 1.33, respectively, in favor of the 10 cc. portion of alkali.

Very little can be said regarding the influence of the quantity of alkali in the case of the meats and urines since the data are too few for consideration. The slight differences in those instances that are reported are inappreciable when calculated to their final percentages. However, in connection with the meat extract the facts show very plainly that the 10 cc. of alkali when used with 15 cc. of picric acid gives higher results than the 5 cc. portion.

In the second trial where 10 and 15 cc. of alkali were used with 30 or 45 cc. of picric acid, the resulting data for the beef extracts and meat are fairly constant. The few variations amount in the maximum to only 0.2 mm. This would indicate that the slight differences were not due to the excess of alkali, but rather

to errors in the technique. However, it should perhaps be stated that these differences are in the main in favor of the lower readings for the 10 cc. test, but when the data in Table 10, relating to pure creatin, are also taken into account the evidence seems to be such that it can be stated that there are no differences resulting in using either 10 or 15 cc., of alkali with 30 cc. of the picric acid, beyond the experimental errors.

From the above consideration, the data show that the quantity of alkali does influence the depth of color, that a small quantity does not yield as high a percentage as a large one; that a large excess does not give low results, and that the accepted 10 cc. portion, yields better results than the 5 cc. and similar results to the 15 cc. portion.

(*b*) *Influence of picric acid.* In the preceding pages, the effect of using varying amounts of alkali and picric acid has been considered where preformed creatinin was to be determined, and in the above paragraphs, the influence of different quantities of alkali has been discussed in the case where both the preformed and dehydrated creatinin were present. It is the purpose of this section to digest the data herein reported which refer directly to the effect of using larger quantities of picric acid than has been customary in determining creatin as creatinin.

Hehner noticed in his work that an increase of the picric acid from 15 cc. to 25 cc. (1.01 per cent) caused a marked difference in the amount of creatinin found to be present in meat extracts. Instead of obtaining with 25 cc. of picric acid (1.01 per cent) 6 to 7 per cent of creatinin, which he got with the equivalent of 15 cc. of 1.2 per cent acid, he reported 10 to 12 per cent. He found, further, that a larger amount of picric acid had no increased effect.

In our work, the amounts of picric acid used were 15, 21, 30 and 45 cc., and since it was found that 10 cc. of the sodium hydroxide worked satisfactorily, this quantity of alkali was taken throughout for the comparison. The data in Tables 2, 4, and 7 on meat extracts, Table 5 on meat, and Table 10 on pure creatin, show that in the case of 30 cc. of the picric acid the general tendency is to produce a lower reading and hence a higher percentage of creatinin as creatin. These differences in the readings for the meat extracts are practically nothing in sample 2309

and 1.4, 0.6, 0.5 and 0.2 mm. in samples 2278a, 2278b, 2307 and 2310, respectively; for the meat they are 0.5 mm., and for the pure creatin 0.1 to 0.6 mm. Concerning the data for the urine, Table 9, the differences due to the increased amount of picric acid are very slight, being in samples 2319 and 2321 almost nothing and in sample 2320, 0.3 mm.

In general then, it may be stated for the meat extracts, meat and pure creatin that the additional quantity of picric acid may have no effect in some cases and in others it may cause a decrease in the readings of 0.2 to 1.4 mm. From the data in the following Table 12, which gives a summary of these facts, the differences

TABLE 12. SUMMARY OF RESULTS ON INFLUENCE OF PICRIC ACID.

(*Creatin.*)

Lab. No.	SAMPLE.	READINGS OF COLORIMETER.			PERCENTAGE OF CREATIN.		
		Picric acid 15 cc.	Picric acid 30 cc.	Difference.	Picric acid 15 cc.	Picric acid 30 cc.	Difference.
		mm.	*mm.*	*mm.*	*p. ct.*	*p. ct.*	*p. ct.*
2278a	Beef extract.....	10.5	9.1	1.4	1.53	2.15	0.62
2278c	" "	8.8	8.2	0.6	1.82	2.10	0.28
2307	" "	6.9	6.4	0.5	1.22	1.57	0.35
2309	" "	7.9	7.8	0.1	1.29	1.36	0.07
2310	" "	5.8	5.6	0.2	2.88	2.96	0.08
2279	Meat.....	7.5	7.0	0.5	0.42	0.45	0.03
2319	Urine.....	8.2	8.1	0.1	0.30	0.30	0.00
2320	"	8.8	8.5	0.3	0.24	0.25	0.01
2321	"	8.9	8.9	0.0	0.16	0.16	0.00
2289a	Pure creatin.....	9.1	8.5	0.6	88.93	95.78	6.85
2289b	" "	9.5	9.0	0.5	89.69	94.18	4.49
2289c	" "	8.9	8.5	0.4	89.45	94.69	5.24
2289d	" "	7.7	7.3	0.4	90.63	95.18	4.55
2289e	" "	9.0	8.9	0.1	94.71	95.24	0.53
2289f	" "	8.4	8.0	0.4	95.80	99.98	4.18

in the percentage of creatin in the meat extracts are seen to vary from 0.07 to 0.62, the total percentage of creatin being 1.4 to 3.0. The meat shows a corresponding difference, a gain of 0.03 per cent on a total of 0.446 per cent. In the case of the urine the variations are practically nothing, and in that of the pure

creatin, they are on the average 4.4 per cent greater for the 30 cc. portion of acid.

A second point should be considered, as to the influence of using still more of the picric acid. The data for the sample of beef extract 2278a, Table 2, and that for the meat 2279, Table 5, show that an additional 15 cc. of acid, or 45 cc. in all, does not cause any further change than that brought about by the 30 cc. test.

The above two facts agree in general with Hehner's conclusions that 25 cc. (1.01 per cent) of picric acid should be used and that an additional amount produces no different effect. However, mention should be made that in our case where 21 cc. of the 1.2 per cent picric acid, which is the equivalent of 25 cc. of a 1.01 per cent solution was used, no decided change was produced. This fact is shown in the data in Table 4. Further, while the additional amount of picric acid seems in general to cause a deeper color, the authors wish to emphasize the fact that this difference is by no means as great as Hehner states it to be in his paper. After calculating the data, obtained by using 30 cc. of picric acid and 10 cc. of alkali, it will be seen that the combined percentages of creatinin and creatin in the six different samples of beef extract reported in Tables 2, 4, and 7 amount to 4.15, 4.11, 6.71, 3.80, 1.63, 5.24 and 6.46. These samples correspond, respectively, to those for Laboratory Nos. 2278a, 2278c, 2306, 2307, 2308, 2309 and 2310. When these percentages are compared with those reported in the previous paper which ranged from 1.38 to 6.56 per cent, it can be stated that the data do not differ materially in the two cases, and in as much as the samples used for this work are both representative of those reported formerly and also of Hehner's the evidence seems to indicate that Hehner's results which varied from 10 to 12 per cent for the combined creatinin and creatin were entirely too high.

From the above consideration of the data, it is evident that in the majority of cases the method gives higher results for the converted creatin in meat extracts, meat and pure creatin when the quantity of picric acid (1.2 per cent) is increased from 15 to 30 cc., although several instances are reported where the smaller amount of acid served equally well. No definite explanation can be given at the present time for this apparent anomaly. The

tendency seems to indicate that it is more difficult for the 15 cc. of picric acid to overcome the resistance of the resulting converted creatinin than that of the preformed creatinin which shows that the former must exist in a different condition than the latter. The amount of hydrochloric acid used in dehydrating the creatin does not seem to influence the results as is shown in Experiment 10 which relates to the pure creatin. Here, some solutions had 90 cc. of hydrochloric acid and others 50 cc., yet the general effect of the picric acid was the same in each case. Jaffe,[1] states that by using the zinc chloride method his maximum yield was 94.83 per cent and adds that this seems to show that the creatinin resulting from converted creatin is broken down to some extent by the strong acid. In general the results reported in this paper confirm Jaffe's conclusion. Benedict and Meyers[2] in using Folin method with creatin obtained from 96 to 98.9 per cent, while the data here reported show a variation in six determinations of from 94.2 per cent to 95.8 per cent, and in one test the result was 99.98 per cent. These facts, however, do not necessarily reflect upon the Folin colorimetric method as applied to urine, meat and meat extract. Since normal urine contains no creatin, meats only 0.44 per cent, and meat extracts from 1 to 6 per cent, it will be seen that a yield of 95 per cent is sufficiently accurate for all practical purposes. Further, in as much as the modified method seems to give uniform results throughout, the data should be comparable in all cases and be of extreme value in giving important information as to the quantity of these extractives in meat products.

OUTLINE OF METHOD AS NOW USED IN THIS LABORATORY.

The following brief outline of the method as now used is given: For the preformed creatinin, transfer aliquot portions of the sample solution to 500 cc. measuring flasks, add 15 cc. of a 1.2 per cent picric acid solution, mix, add 10 cc. of a 10 per cent sodium hydroxide solution, shake thoroughly, and allow the mixture to stand 5 minutes and then dilute to the mark at once and after mixing, compare the depth of the color of the solutions with that

[1] *Zeitschr. f. physiol. Chem.*, xlviii, p. 436, 1906.
[2] *Amer. Journ. of Physiol.*, xviii, p. 4 1907.

of a half-normal bichromate solution set at 8 mm. According to Folin, the correct reading in millimeters of the colorimeter divided into 81 gives the number of milligrams of creatinin contained in the portion of the solution taken for the treatment with picric acid and sodium hydroxide. In other words, 10 milligrams of pure creatinin after the addition of the picric acid and the alkali and dilution to 500 cc., gives a reading of 8.1 mm. when compared with 8 mm. of $\frac{N}{2}$ potassium bichromate solution (24.54 grams per liter). For the combined creatinin, transfer aliquot portions of the sample solution to beakers, if the quantity is more than 10 cc., or to 100 cc. measuring flasks, if the quantity is 10 cc. or less. In the former instance, evaporate the solution on the water-bath to 10 cc. In either case make the volume of the liquid up 10 cc., if necessary, and add 10 cc. of normal hydrochloric acid. Rotate the vessels to mix the liquids. Transfer the acid solutions to an autoclave and heat them at a temperature of 117 to 119° C. for 30 minutes. After removal, cool and dilute to the mark. If beakers were used, transfer the contents to 100 cc. measuring flasks and dilute. To aliquot portions of the converted creatinin solution add, in 500 cc. flasks, 30 cc. of 1.2 per cent picric acid, shake and then add 10 cc. of the 10 per cent sodium hydroxide. Mix thoroughly and after standing exactly 5 minutes dilute, and read the depth of color of the solution. In order to convert milligrams of creatinin into creatin multiply by the factor 1.16.

It was found that by using a black cloth, to shut out the surrounding light from the eyepiece of the instrument, the colors appeared more distinctly, and that the comparison could be made more accurately and rapidly and with less strain on the eye.

CONCLUSIONS.

From this study upon meat extracts and meat, the following conclusions can be made in regard to the applicability of the Folin method for determining creatinin and creatin:

(*a*) That an increase in the quantity of picric acid, according to Hehner's suggestion causes no difference in the so-called original creatinin determinations; but it generally does produce an appreciable difference when the converted creatin is also

present, and, further, that the quantity of picric acid (1.2 per cent) recommended for use in meat extract, meat and urine should be left at 15 cc. for the original creatinin determinations and be increased to 30 cc. for the dehydrated creatinin.

(*b*) That in the determination of the original creatinin, the use of a small or large amount of 10 per cent alkali makes almost no difference, the 5 cc. quantity giving slightly lower results than the 10 and 15 cc. quantities; that, for the converted creatin, the previously accepted quantity of alkali, 10 cc., gives better results than 5 cc. and equally as good results as the large excess, 15 cc.; and further, that these facts are contrary to those found by Hehner who states that a quite small amount of alkali gives better results than a large quantity which he maintains diminishes the depth of color.

(*c*) That the data reported are representative of the percentages of creatinin and creatin in meats and meat extracts, being practically the same for the combined extractives as those previously published, 0.45 per cent for the former and 1.4 to 6.5 per cent for the latter, whereas Hehner found the total percentage of creatinin and creatin in meat extracts to be 10 to 12 per cent.

(*d*) That the Folin method when properly modified is as applicable to meat extracts and meats as it is to urine, and that it gives reliable and concordant results in the hands of different analysts of this laboratory.

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ERRATA, VOLUME III.

Page 85, line 26, for *exclusive* read *extensive*.

Page 90, line 7, for *chloride* read *hydroxide*.

Page 180, Nos. (3), (4), (5) and (6) of table of acetone determinations should read, "20 cc. acetone solution + 0 cc. H_2O, etc."

INDEX TO VOLUME III.